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Axonal and synaptic pathology in Alzheimer's disease

by

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Summary

The cause of the initial synaptic disconnection and eventual widespread neuronal degeneration that underlies the onset and progressive development of dementia in sufferers of Alzheimer's disease (AD) remains elusive. The pathognomonic features of AD, extracellular accumulations of soluble and fibrillar β-amyloid (Aβ) as well as intracellular neurofibrillary tangles comprised of hyperphosphorylated tau, that give rise to characteristic dystrophic neurites and neuropil threads, respectively, have been studied extensively in human AD cases and a variety of transgenic mouse models. Nonetheless, the degree to which these malformations affect different populations of neurons and their synaptic connections in the cortex remains to be defined. Furthermore, although white matter degeneration has previously been implicated in AD, not much is known about the extent of myelin loss in AD. This thesis, therefore, sought to address four aims analyzing the relationship between AD pathology and the mechanisms underlying AD. Firstly, to investigate the extent to which interneuron subpopulations are susceptible to Aβ plaque-mediated cytoskeletal alterations compared to a neurofilament-rich pyramidal neuron population. Secondly, to examine the relationship between Aβ plaque deposition and inhibitory and excitatory synaptic connections. Thirdly, to assess if the activity of glutamate decarboxylase, the enzyme catalysing the formation of the inhibitory neurotransmitter GABA, is altered in a transgenic mouse model of AD. Finally, to determine if AD pathology is associated with cortical demyelination and oligodendrocyte cell loss in human and transgenic mice.

The major conclusions drawn from these investigations were that inhibitory interneuron neurites were not as susceptible to Aβ plaque-mediated dystrophy as

neurofilament-rich neurites. Moreover, GABAergic synaptic density was not significantly decreased in proximity to Aβ plaques unlike excitatory glutamatergic synapse density. These decreases were accompanied by potentially compensatory changes in presynaptic bouton size, perisomatic innervation, as well as increased gliotransmission of GABA in Aβ plaque-rich neuropil. Neuritic plaque deposition was also associated with focal demyelination and concomitant decreases in several integral myelin-associated proteins. Interestingly, although mature oligodendrocyte loss was also present, there were significant increases in the number of immature oligodendrocytes and precursor cells, indicative of a reactive remyelinating response. In summary, this thesis further clarified the pathological role of Aβ plaques in mediating cytoskeletal dystrophic changes and specific synaptic loss. It also identified the novel finding of focal demyelination associated with Aβ deposits. A better understanding of these early pathological alterations in the progression of AD is necessary for the development of effective therapeutic strategies. In particular, the compensatory changes in response to ongoing AD pathology could offer promising endogenous targets for slowing or repairing neuronal dysfunction.

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TABLE OF CONTENTS

Abbreviations:

AD	Alzheimer's disease
APP	Amyloid precursor protein
AZ	Active zone
Aβ	β-Amyloid
CB	Calbindin D-28
CNS	Central nervous system
CR	Calretinin
DN	Dystrophic neurite
fAD	Familial Alzheimer's disease
GAD	Glutamate decarboxylase
-ir	Immunoreactive
NFs	Neurofilament triplet proteins
NFT	Neurofibrillary tangles
OPC	Oligodendrocyte precursor cell
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PHF	Paired helical filament
PRE	Preclinical Alzheimer's disease
PS	Presenilin
PSD	Post-synaptic density
PV	Parvalbumin
SDS	Sodium dodecyl sulphate
SV	Synaptic vesicle
SYN	Synaptophysin
TG	Transgenic mouse
VGAT	Vesicular GABA transporter
VGlut	Vesicular glutamate transporter
WT	Wildtype mouse

1. Introduction

In 1906, Alois Alzheimer presented the case of 51 year old Auguste D, describing in detail the typical clinical characteristics with the attendant memory deficits, as well as the neuropathological features he described as 'military bodies' (plaques) and 'dense bundles of fibrils' (tangles) that we today commonly recognize as the chief hallmarks of the eponymously named disease (Möller and Graeber 1998). Since its obscure beginnings over a century ago, Alzheimer's disease (AD) has become the most common type of neurodegenerative disorder and the leading cause of dementia worldwide, accounting for more than 70% of all cases (Alzheimer's Association 2012; Sosa-Oritz et al. 2012). Despite a concentrated research effort spanning decades, there is still no complete understanding or effective treatment of the underlying pathology of AD (Golde et al. 2011; Huang and Mucke 2012). Epidemiological studies suggest that, aside from rare mutations that give rise to the familial form of the disease, age is the principal risk factor for AD as less than 1% of cases occur in individuals below 65 years (Sosa-Oritz et al. 2012; Bettens et al. 2013). The incidence of the disease doubles for every 5 years thereafter, increasing to 6-8% for those aged 65 to 70, followed by an exponential rise to 24-33% in the population aged over 85 (Mayeux and Stern 2012).

However, data in centenarians suggests that AD is not an inevitable outcome of aging; rather, it could be a disease process that is superimposed on the background of, and is compounded by, the concomitant general decline in cognitive function that is a normal part of aging (den Dunnen et al. 2008). Currently, it is estimated that 24.3 million people world-wide have AD and this figure is expected to double in the next 20 years as unprecedented improvements in life expectancy and geriatric care, as

well as the aging 'baby boomer' generation, have created a large aging population (Mayeux and Stern 2012). The incidence of AD is higher in females than in males, but this may reflect longer life expectancy in general and, thus, increased probability of developing AD in older age (Sosa-Oritz et al. 2012).

Although AD develops differently in every patient, there are many common symptoms. At early stages of the disease, the most common symptom is difficulty with short term memory of recent events (Waldemar et al. 2007). This usually confounds early diagnosis as such phenomena are part and parcel of normal healthy aging. There is usually a long insidious stage of disease progression during which most individuals are largely asymptomatic or only exhibit mild impairments in cognition. As AD develops, more obvious symptoms can include confusion, irritability and aggression, mood swings, trouble with language, and finally, long-term memory loss (Warren et al. 2012). In the final stages, bodily functions are usually lost, ultimately resulting in death. On average, the life expectancy following diagnosis is approximately seven years. As multiple factors such as education, socioeconomic status, genetics, diet, and physical activity can affect AD time course and severity, it is often difficult to establish individual prognosis (Schupf et al. 2005; Scarmeas et al. 2007; Roe et al. 2008).

1.1 Pathological hallmarks of AD

Alzheimer's disease is characterized by extracellular accumulations of β-amyloid (Aβ), a secreted protein of unknown function which undergoes an abnormal conformational change causing it to aggregate into insoluble fibrils in the neuropil (Busciglio et al. 1993; Haass and Selkoe 1993; Citron et al. 1995). Microscopically,

Aβ deposits appear as plaques which may have a variety of morphological forms such as spherical diffuse, fibrillar and dense-cored based on the density of their constituent Aβ fibrils (Dickson and Vickers 2001; Adlard and Vickers 2002; Bussiere et al. 2004). These are accompanied by intraneuronal protein inclusions such as neurofibrillary tangles (NFTs) and neuropil threads that are largely composed of abnormally hyperphosphorylated tau protein and other cytoskeletal elements (Iqbal and Iqbal 2008). β-Amyloid plaques have been proposed to have toxic (Pike et al. 1993; Busciglio et al. 1995; Pike et al. 1997; Garcia-Alloza et al. 2006) and/or physical 'mass' effects (Vickers et al. 2000; Dickson and Vickers 2001; Adlard and Vickers 2002; Tsai et al. 2004; Dickson et al. 2005; Woodhouse et al. 2005) on the surrounding neuropil, ultimately causing gross cytoskeletal changes that lead to widespread neuron loss.

The prevalence and distribution of both types of lesions, intraneuronal NFTs and extracellular plaques, extends progressively throughout the disease, encompassing the neocortex, hippocampal formation and subcortical regions, and is accompanied by severe grey matter atrophy (Braak et al. 2011). The most atrophic areas of the cerebral cortex are the medial and inferior portions of the temporal lobe, followed by the posterior and inferior parietal lobe, and to a lesser extent, portions of the anterior and superior frontal lobe. Atrophy is mostly correlated with NFT pathology (Braak et al. 2006), and to a lesser extent, with neuritic plaque-mediated degeneration (Halliday et al. 2003). This has led some to suggest that the number of NFTs, which increases progressively and consistently throughout disease process, as well as their distribution (entorhinal/perirhinal cortex and hippocampus affected earlier while neocortex is affected later) are more reliable correlates of cognitive decline in AD (Arriagada et al. 1992; Giannakopoulos et al. 2003). However, more recent imaging

studies in AD patients (Hedden et al. 2009; Lim et al. 2012), combined with *in vivo* data from transgenic mouse models (Myer-Luehmann et al. 2008; Bittner et al. 2012), have challenged this view, providing evidence that neuritic plaques do in fact correlate with clinical dementia rating scores (Parvathy et al. 2001) and that plaque deposition precedes NFT formation (Oddo et al. 2007).

Nevertheless, a recent analysis of 42 post-mortem brains from non-demented individuals aged 14 to 29 revealed that nearly all had some degree of abnormal tau phosphorylation even at this early stage, while only one case showed signs of extracellular amyloid deposition (Braak and Del Tredici, 2011). Although only three cases showed isolated true NFT-type pathology in the transentorhinal region, this study shows for the first time that pathological processes associated with AD, particularly NFTs, may begin as early as adolecense in some individuals. Assuming that these 'pre-tangle' lesions are not transient and do not regress over time, this has serious ramifications for the current understanding of AD as an ageing-related pathological process. One potential criticism of this work however, is that Braak and Del Tredici (2011) did not consider intracellular Aβ in their analysis of early pathological changes. Intracellular Aβ has been shown to occur early in disease progression in familial AD (Rovelet-Lecrux et al. 2006; Kasuga et al. 2009), sporadic AD (Gouras et al. 2000; D'Andrea et al. 2001, 2002), Down's syndrome (Gyure et al. 2001), as well as numerous transgenic mouse models (reviewed in LaFerla et al. 2007), potentially preceding the deposition of plaques and formation of hyeprphosphorylated tau-bearing tangles. Regardless of the nature of the pathology, be it thangles or plaques, the functional significance of such early alterations are not entirely clear; further research, especially long-term imaging combined with

biomarker and cognitive testing in young to middle-aged individuals could provide a better understanding.

The hippocampal formation is also severely affected in AD. Subregions that are critical for both cortical and subcortical efferent projections, such as the cornu Ammonis region 1 (CA1) and subiculum, accumulate the most NFT pathology while the neighbouring CA3-CA4 regions are relatively spared (van Hoesen and Hyman 1990; Price et al. 2001; Scheff et al. 2007). Hippocampal input is also severely compromised as layer 2 of the entorhinal cortex, responsible for most of the hippocampal afferents such as the perforant pathway, is severely affected by neurofibrillary alterations early in the disease (Hyman et al. 1990; Arnold et al. 1991; Gomez-Isla et al. 1996). This disruption of the extrinsic and intrinsic hippocampal circuitry leads to the structural and functional loss of cortico-cortical and cortico-limbic connections that are associated in memory and higher cognitive function, allowing for the ensuing dementia and memory deficits characteristic of AD (Braak et al. 2011). Frank dementia is thought to be preceded by a long, insidious 'preclinical' phase characterized by increased plaque deposits and neurofibrillary changes but no overt clinical symptoms (Price and Morris 1999; Morris et al. 2001).

Histopathologically, the cytoskeletal alterations associated with AD present as follows: i) dystrophic neurites (DNs) are misshapen neuronal processes, likely of axonal origin that are localized within and around plaques, ii) neuropil threads are proteinaceous accumulations believed to be of dendritic origin, and iii) NFTs comprised of paired helical tau filaments and occuring in the soma (Braak and Braak, 1988). In contrast to Aβ plaques, NFTs, DNs and neuropil threads consist of pathologically altered intraneuronal filamentous (neurofibrillary) accumulations of cytoskeletal and cytoskeletal-associated proteins (Spillantini et al., 1996;

Giannakopoulos et al., 2003). DNs, in particular, can be immunoreactive for phosphorylated and dephosphorylated neurofilament triplet proteins (NFs), amyloid precursor protein (APP), ubiquitin, α-internexin, and/or tau (Masliah et al. 1993; Su et al. 1996; Vickers et al. 1996; Su et al. 1998; Thal et al. 1998; Dickson et al. 1999; Dickson and Vickers 2001; Dickson et al. 2005). DNs may also contain large, abnormal accumulations of pre- and post-synaptic proteins such as synaptophysin, synapsin and PSD-95 among others, suggesting that this type of pathology is very closely associated with synaptic dysfunction in AD (see section 1.5; Masliah et al. 1994; Brendza et al. 2003; Sanchez-Varo et al. 2012). Not all Aβ plaques are associated with DNs, but clusters of DNs are always associated with a characteristic subset of plaques (Noda-Saito et al. 2004). This latter subpopulation of Aβ deposits, known as 'neuritic plaques', is mostly comprised of more compact/fibrous type of plaques, consistent with a potential mass-related physical effect of more dense plaques (i.e. 'pushing' against, or compression of surrounding neuropil) (Dickson et al. 1999; Woodhouse et al. 2005).

Morphologically, DNs present as swollen tortuous neurites 10-60 μm in diameter with variable shapes and composition depending on the pathological stage of AD (Masliah et al.1993; Vickers et al. 1996; Dickson and Vickers 2001; Dickson et al. 2005). In preclinical AD cases as well as in Tg2576 and TgCRND8 transgenic mice, Aβ plaque-associated DNs are frequently labelled with antibodies to NFs and α-internexin, but not for the abnormally hyper-phosphorylated tau protein that characterizes NFT pathology in end-stage AD cases (Benzing et al. 1993; Su et al. 1998; Dickson et al. 1999; Woodhouse et al. 2009b). These DNs usually include bulb-like swellings and ring-like spherical structures with a hollow core that may be continuous with an axon or appear as isolated entities (Dickson and Vickers 2001).

In contrast, in end-stage AD, particularly familial types, there are abundant tau-labelled DNs that appear as angular, elongated structures (Su et al. 1996; Dickson et al. 1999; Woodhouse et al. 2009a). There also numerous NF- and α-internexin-immunolabelled bulbous and sprouting DNs (Dickson et al. 2005). A large proportion of these NF-labelled DNs exhibit a previously absent core of tau protein immunoreactivity (Su et al. 1998; Dickson et al. 1999). This suggests that DNs may mature from mostly NF/α-internexin-expressing DNs, to DNs that express predominantly tau as disease progresses. Evidence for such time-dependent maturation of cytoskeletal abnormatlities has also been found in APP/PS1, Tg2576 and TgCRND8 transgenic mice where DNs positive for phosphorylated NFs were found several months before hyper-phosphorylated tau-immunolabelled DNs appeared (Masliah et al. 1991; Blanchard et al. 2003; Woodhouse et al. 2009b). Such staging of cytoskeletal alterations may have important implications for progression of NFT pathology in neurons.

1.2 Genetics of AD

Genetically, Alzheimer's disease is a heterogeneous disorder with an autosomal dominant early-onset familial form (fAD) and a late-onset sporadic form (sAD). The sporadic form is by far more common, accounting for more than 90% of all reported AD cases (Bettens et al. 2013). Both the sporadic and familial forms of the disease result in the accumulation of β-amyloid (Aβ) in the cortex, which is believed to drive the pathogenesis of AD. Familial mutations in APP and the presenilin family of genes (PS-1 and PS-2) that comprise part of the multimeric γ-secretase complex, may increase the production of pathogenic forms of Aβ via different mechanisms that are summarized in Table 1. Missense mutations in the APP gene were the first to be

discovered but they only account for a small fraction (~0.1%) of all fAD cases (O'Brien and Wong 2011). Presenilin mutations, on the other hand, account for the vast majority of fAD mutations with over 150 currently identified (De Strooper 2007). Although fAD cases only account for small percentage of all AD diagnoses, their study has nevertheless been very useful for deciphering the biochemical signalling pathways involved in AD through the development of transgenic AD mouse models expressing various combinations of these mutant genes.

1.2.1 Amyloid Precursor Protein

The APP gene on chromosome 21 is approximately 400kb long with 19 coding exons and is physiologically expressed by both neuronal and non-neuronal cells (Busciglio et al. 1993; Koo et al. 1990). The variously spliced mRNAs give rise to several APP isotypes (770AA being the longest variant), most notably the 695AA form which is expressed by neurons (Koo et al. 1990). Cleavage by α-secretase at residue 687 within the single transmembrane Aβ domain of APP precludes the production of toxic Aβ species and generates two metabolites. One is the large, soluble ectodomain (sAPP-α) that is secreted, whereas the 83-residue C-terminus fragment is retained in the membrane. This latter fragment then undergoes further cleavage by γ-secretase at residue 711 or 713 and a $p3_{40}$ or $p3_{42}$ peptide is secreted (Haass et al. 1992; Citron et al. 1995). If, however, β-secretase acts upon APP instead of α-secretase, the resultant metabolites are a slightly shorter sAPP-β (cleaved at residue 671) and the retention of the entire transmembrane Aβ domain (99-residue C-terminus fragment). This C99 fragment then undergoes cleavage by γ-secretase at residue 711 or 713 to give rise to the pathogenic $Aβ_{1-40}$ and $Aβ_{1-42}$ peptides. All of the mutations that result in fAD are clustered either at or near the various secretase cleavage sites. These alterations in the APP gene can lead to AD in two main ways: i) overexpression of APP with

general increases in all Aβ species (analogous to the gene-dosage effect in trisomy 21 Down syndrome) such as APP gene locus duplication seen in Caucasian (Rovelet-Lecrux et al. 2006) and Japanese carriers (Kasuga et al. 2009), or ii) mutations that increase the amyloidogenic cleavages of APP at either the β-secretase or γ-secretase site that result in selectively increasing the production of $A\beta_{1-40}$ and $A\beta_{1-42}$. Support for the latter, particularly for the critical amyloidogenic role of β-secretase cleavage, has recently come from a report of a protective APP mutation in an Icelandic population study (Jonsson et al. 2012). The authors found that the A673T substitution that ocurrs adjacent to the β-secretase cleavage site, results in a 40% reduction in amyloid peptides *in vitro* as well as improved cognitive ability in non-demented carriers. This study provides proof of concept that therapeutic strategies aimed at reducing β-secretase activity could have a beneficial effect on both healthy aging and in AD.

1.2.2 Presenilins

In contrast to the APP mutations, the PS-1 and PS-2 mutations account for a greater proportion of fAD cases, causing an earlier and more aggressive form of AD, usually beginning between age 40 and 60 (earlier than the APP-related mutations) and leading to a higher morbidity and mortality (De Strooper 2007). The PS-1 and PS-2 genes code for homologous, polytopic transmembrane proteins that form the catalytic part of the multiprotein γ-secretase complex (De Strooper 2003). Mutations are mostly segregated to the transmembrane regions of the PS protein and appear to result in an alteration in APP processing that selectively increases the production of $A\beta_{1-42}$ peptides (Selkoe and Wolfe 2007). More recently, other physiological substrates of γ-secretase cleavage such as Notch-1 have been discovered and it has been shown to play a crucial part in neuronal calcium regulation (Zhang et al. 2010),

neurogenesis (Gadadhar et al. 2011), as well as oligodendrocyte differentiation (Watkins et al., 2008).

1.2.3 ApoE and other genetic risk factors

Other genes have been identified that are not necessarily associated with fAD directly, but increase the susceptibility to AD and the associated morbidity in both familial and sporadic cases. Inheritance of polymorphisms in the gene coding for apolipoprotein E (ApoE), the ε4 allele in particular, have been found to lead to an earlier disease onset and faster progression in a gene dose-dependent manner (homozygotes for ApoE ε4 allele being the worst affected) (Schmechel et al. 1993; Strittmatter et al. 1993). Apolipoprotein E is expressed constitutively as part of the lipoprotein family of cholesterol transporters and has an important function in repair and maintenance of neurons (Mahley and Young 2013). Due to a change of residues at a key location (C112R), the N- and C-terminals of ApoE (ε4) interact which reduces the stability and functional capacity of the protein (Kim et al. 2009).

Furthermore, ApoE may increase the aggregation of Aβ and is localized to neuritic plaques that are associated with DNs (Dickson et al. 1997). The ε4 allele is also associated with a reduction in the functional capacity of ApoE in the clearance of Aβ from the neuropil, as well as a potentiation of Aβ-induced lysosomal leakage leading to neuronal apoptosis (Mahley et al. 2006). The reduced ability to perform its physiological roles along with an associated increase in the pathogenicity of Aβ, render ApoE ε4 an important susceptibility factor for sporadic AD (Bu 2009).

Linkage studies have also recently implicated chromosome 10 as a putative disease locus with the gene coding for insulin degrading enzyme (IDE) as the candidate gene (Bertram et al., 2000). Vekrellis and colleagues (2000) showed that IDE is not just a

metalloendopeptidase involved in the degradation of various peptide hormones, but is also secreted by microglia and is involved in the degradation of extracellular Aβ. This finding also explains why patients with hyperinsulinaemia are at higher risk of developing AD, since insulin competes with Aβ for the proteolytic actions of IDE (Luchsinger et al., 2004). Genome-wide association studies have provided further evidence for genetic linkages to late-onset AD. A study by Rogaeva and co-authors (2007) identified the SORL1 gene that codes for a membrane trafficking protein involved in endosome recycling as a likely causal agent.

1.2.4 Mouse models of AD

The identification of the various familial mutations associated with AD has made it possible to design transgenic animal models over-expressing specific genetic factors implicated in the disease process. The advent of transgenic mouse models expressing one or more human fAD-related genes ushered in a new era of molecular and therapeutic research as these models can recapitulate some of the pathological hallmarks and associated cognitive deficits characteristic of AD that the initial human APP-overexpressing models could not (McGowan et al., 2006). Although animal models have proven invaluable in elucidating the multi-faceted nature of AD and identifying novel therapeutic approaches, some of which have led to clinical trials, the fact that they are after all, models, warrants a few important caveats. There is a profound effect of the host animal's background strain on the phenotypes induced by APP mutations which has implications for analysis and comparison with humans (Carlson et al., 1997; Bussiere et al., 2004). Other differences arise from the mutations that the models express; for example, in Tg2576 mice, the $APP_{(Swe)}$ double mutation increases $A\beta_{1-42}$ and to a lesser extent, $A\beta_{1-40}$ levels (Hsiao et al., 1996), resulting in a mostly dense-core plaque phenotype with very few diffuse plaques. In

contrast, diffuse plaques predominate in the PDAPP model that selectively increases $A\beta_{1-42}$ levels only (Games et al., 1995).

It is noteworthy that most of the mouse models differ in their histopathological presentation from human AD cases: none of the models develops any signifcant degree of neuron loss and only some develop neurofibrillary pathology, the latter by also over-expressing a human tau mutant that is normally associated with familial frontotemporal dementia (Games et al. 1995; Oddo et al. 2003). A further complication is the assessment of AD-like cognitive deficits. Many of the canonical diagnostic features of AD involve loss of functions that are uniquely human such as deficits in language and verbal episodic memory that cannot be replicated in transgenic mice (Chapman et al. 2001). Furthermore, because these mice overexpress mutant forms of human APP and/or PS1 under constitutively-active promoters such as PrP/Thy1 and not the mouse APP promoter, endogenous APP gene regulation that may result in gender differences in Aβ accumulation and deposition in humans (Sosa-Oritz et al. 2012) may be obscured in mice. However, the profound loss of memory that is the earliest and most recognizable feature of AD allows for spatial learning and memory tasks (e.g. Morris water maze) to be used as diagnostic markers when assessing cognitive decline in transgenic mice. When drawing conclusions from model-based studies, it is also crucial to distinguish between age-dependent and age-independent features, the latter of which occurs as a result of over-expression of APP during development, while the former arises from biochemical or structural changes as mice age and accumulate AD-related pathology (Westerman et al. 2002).

The Tg2576 line is perhaps the most widely-used APP overexpressing model and harbours cDNA with hAPP that has the double Swedish mutation under the control

of the hamster prion protein promoter (*PrP*) (Hsiao et al. 1996). AD pathology appears in a time-dependent manner, with the first plaques developing around 7-9 months of age, with more significant plaque loads around 12-15 months of age (Kawarabayashi et al. 2001), leading to neuritic changes and gliosis but no overt neuron or synapse loss in CA1 and dentate gyrus (Irizarry et al. 1997). More recently, this has been challenged by Tomidokoro and colleagues (2001) who argued that neurons are displaced by dense-core plaques and that previous stereological studies looked at total amyloid burden only, not accounting for the dense-core plaque-specific effect. The onset of plaque deposition also coincided with age-dependent cognitive deficits such as impaired *in vitro* and *in vivo* long-term potentiation (LTP) in the hippocampus (Chapman et al. 1999) and a disruption in synaptic integration of transcallosal stimuli (Stern et al. 2004). Interestingly, overall plaque load and concentrations of Aβ1-42/1-40 are much higher in aged (15-19 month) female Tg2576 mice than males (Callahan et al. 2002; Hirata-Fukae et al. 2008), which parallels the human condition.

Dendritic spine density is also decreased in, and immediately around, dense-cored plaques in Tg2576 mice, demonstrating that these models recapture some of the loss of synaptic circuitry in AD (Le et al. 2001; Lanz et al. 2003; Spires et al. 2005). Jacobsen and colleagues' (2006) study on the temporal pattern of progression of neuronal deficits in Tg2576 mice demonstrated that AD-like changes can be clustered into two temporally distinct phases. Significantly, in the early phase (4-5 months), there was a decrease in dendritic spine density with deficits in neurotransmission and LTP in neurons of the dentate gyrus in the absence of Aβ plaque deposition. The observation that these changes occurred well before any plaques were observed in this transgenic line prompted the authors to conclude that:

i) dysfunction of the circuitry of the outer molecular layer of the dentate gyrus is one of the earliest anatomical effects of APP overexpression, and, ii) that these early-onset morphological and functional changes are mediated by one or more soluble pre-plaque Aβ oligomers (Jacobsen et al. 2006).

As mutations in the two presenilins were identified and shown to be responsible for the bulk of FAD cases (De Strooper 2007), the need for mouse models that replicated the effects of these mutations became apparent. Duff and colleagues (1996) were the first to construct a PS-1 transgenic mouse model which showed that overexpressing human PS-1 protein bearing the M146L and M146V FAD-associated mutations, but not wildtype PS-1, led to an increase in $A\beta_{1-42}$ levels in the brain. As mutations in the presenilin genes play an integral role in the APP processing pathway that leads to Aβ formation in FAD, several groups have made PS deficient mice. Shen and colleagues (1997) showed that PS-1 knockout mice developed a very severe phenotype resulting in embryonic lethality due to the importance of PS-1 mediated Notch signalling pathways during development. PS-2 knockout mice proved viable but displayed no visible changes in APP processing, showing that there was a degree of redundancy in some PS-1 and -2 function (Herreman et al., 1999). Finally, PS-1 conditional knockout mice, in which inactivation is postnatal, showed a significant decrease in Aβ generation with a subtle deficiency in LTP, suggesting that treatments targeting γ-secretase could prove useful, as long as they did not perturb its APP-independent functions (Yu et al., 2001).

Crossing the Tg2576 mice with PS1 M146L mice gave rise to the now widely used APP/PS1 mouse model (Holcomb et al., 1998). These transgenic mice develop plaques within 16 weeks as opposed to 7 months as seen in the Tg2576 line alone. Although larger numbers of fibrillar plaques have been reported in this model (Le et

al. 2001), there is a lack of neuron loss in the hippocampus and cortex (Takeuchi et al. 2000). Cognitive deficits have been reported as early as three months, before the onset of plaque deposition (Holcomb et al., 1999) although these deficits did correlate well with increased plaque burden at later ages (Trinchese et al. 2004). There are now many other types of transgenic AD model mice that harbour additional mutations in the tau gene as well (e.g. Oddo et al. 2003), however, for the scope of this thesis, they will not be discussed (for an excellent review of the topic, see Crews et al. 2010).

1.3 The 'Aβ hypothesis' of AD

Fundamentally, the amyloid hypothesis holds that aberrations in APP processing result in increased production of Aβ and its aggregation into Aβ plaques due to an interplay between genetic and environmental factors, causing a pathological cascade of cytoskeletal changes leading to widespread neurodegeneration and synaptic loss with ensuing dementia (Hardy and Selkoe 2002). Evidence for this has come largely from genetic studies showing that mutations in APP lead to an increase in Aβ production and/or enhanced fibril formation and toxicity (Bertram et al. 2010). However, since plaque load is poorly correlated to the degree of dementia in some patients (Arriagada et al. 1992), some groups have suggested the opposing view that accumulation of NFTs drives the formation of plaques and the cognitive impairment in AD (Joseph et al. 2001; Giannakopoulos et al. 2003). More recently, soluble oligomers of Aβ have also entered the debate with a putative role as the principal pathogen driving the disease process (Cleary et al. 2005; Shankar et al. 2008; McDonald et al. 2010).

There are now several lines of evidence that establish Aβ, in its soluble or fibrillar forms, rather than NFT pathology, as playing a critical early role in the cascade of AD pathological changes. The first and most prominent is the finding that plaque formation occurs prior to NFTs (Oddo et al. 2003, 2007; Meyer-Luehmann et al. 2008). A recent 2-photon live-imaging study by Meyer-Luehmann et al. (2008) showed conclusively for the first time that plaque formation preceded and led to secondary neuritic changes (dystrophy) in three different transgenic mouse models of AD. Secondly, Aβ has been observed to have a direct effect on NFT pathology (Busciglio et al. 1995; Gotz et al. 2001; Lewis et al. 2001). Direct intracranial injection of $A\beta_{1-42}$ has been shown to exacerbate NFT-mediated damage in transgenic mice expressing the mutated human (P301L) tau (Gotz et al. 2001). Similarly, crossing P301L mice with Tg2576 mice resulted in an increase in NFT numbers and a wider regional spread than seen in P301L mice alone (Lewis et al. 2001). Finally, SantaCruz and colleagues (2005) have demonstrated that NFTs alone are not sufficient to drive neuronal death and cognitive decline in a mouse model of tauopathy. It remains unclear, however, whether soluble Aβ oligomers or insoluble Aβ plaques are the primary causative agent of neurotoxicity in AD.

Soluble Aβ oligomers

Observations of plaque-independent disruption of neural circuits, decreases in LTP before plaque deposition, and behavioural and synaptic deficits in the absence of Aβ plaques in transgenic mouse models of AD have led to the hypothesis that soluble Aβ oligomers are the main toxic agent in AD (Hsia et al. 1999; Mucke et al. 2000; Westermann et al. 2002; Lanz et al. 2003; Oddo et al. 2003; Reilly et al. 2003; Jacobsen et al. 2006; Shankar et al. 2008; see also Section 1.5). This is further supported by immunotherapy studies which have demonstrated that oligomeric-Aβ

antibody administration leads to a decrease in plaque deposition and tau pathology, resulting in a restoration of cognitive function in mice that were previously impaired (Kotilinek et al. 2002; Oddo et al. 2006). Oddo and colleagues (2006) have also demonstrated that Aβ oligomers are first accumulated intracellularly, and have postulated that these Aβ species represent metastable entities, as opposed to transient precursors of fibrillar Aβ, that are bioactive within neurons, interfering with both intracellular and extracellular (synaptic) function.

Inoluble Aβ deposits

Studies in transgenic mice have also yielded support for insoluble Aβ deposits as drivers of AD pathology. *In vivo* imagining in transgenic mice has revealed that fibrillar Aβ deposits generate reactive oxygen species that are likely to cause oxidative stress in the surrounding neurites (McLellan et al. 2003; Garcia-Alloza et al. 2006b). Indeed, numerous studies have reported that dystrophic neurite alterations such as disrupted neurite trajectories and reduced dendritic spine density are exacerbated in proximity (~50μm) to plaques (Knowles et al. 1999; Moolman et al. 2004; Tsai et al. 2004; Spires et al. 2005). This is accompanied by a robust loss of spines not immediately adjacent to plaques, implying a more widespread loss of postsynaptic density and presynaptic elements supported by a decrease in synaptophysin (a presynaptic protein) immunostaining (Grutzendler et al. 2007). Dendrites traversing plaque-rich neuropil are more likely to have higher intracellular calcium concentrations than those in plaque-free neuropil (Kuchibhotla et al. 2009). Furthermore, there is dendrite loss and increased curvature of neurites within and around dense-cored plaques that extends proximal and distal to the DN, suggesting that these cytoskeletal alterations can be propagated to an extent (Le et al. 2001; Adalbert et al. 2008). This may in turn impair global cortical function, if signal

propagation is slowed down due to the alterations in neuronal geometry, leading to a disruption in synchrony of convergent inputs and a failure to integrate and transmit information effectively (Stern et al. 2004). Also, an expanding body of evidence suggests that plaques may exert a mass-related effect on the surrounding neuropil (e.g. increasing neurite curvature), in addition to their previously postulated toxic effects, making the premise that plaques are inert, protective sinks for the more toxic soluble oligomers, unlikely (Dickson and Vickers 2001; Adlard and Vickers 2002; Woodhouse et al. 2005).

Combined Aβ toxicity?

There is now evidence stemming from *in vivo* and *in vitro* studies that both soluble and insoluble conformations of Aβ are harmful in their own right (Shankar et al. 2008; Knafo et al. 2009; Bittner et al. 2012). This suggests that there may be a 'combined' effect in their pathogenicity (McGowan et al. 2006). Moreover, the two pathways need not be mutually exclusive in driving AD pathology. It had been previously accepted, based on *in vitro* protein aggregation assays (Jarrett et al. 1993) and the observation that it takes months for many plaques to appear even in accelerated mouse models of AD (Oakley et al. 2006), that Aβ follows a relatively slow, time-dependent nucleation polymerization process. Plaque formation has now been shown to take as little as 24 hours *in vivo* and that the associated neuritic changes follow within a week (Meyer-Luehmann et al. 2008). The seemingly contradictory finding that Aβ plaque formation is an acute event could perhaps be explained by the presence of soluble oligomeric Aβ precursors. Presumably, if these plaques form quicker than previously believed, it may be due to the availability of a large enough local concentration of soluble oligomeric Aβ precursors that then precipitate into plaques (Roychaudhuri et al. 2009). Koffie and colleagues have

shown that in both mouse (2009) and human brains (2012) fibrillar plaques are surrounded by a halo of soluble oligomers, suggesting the two entities could be in a dynamic equilibrium.

1.4 Cytoskeletal pathology in AD: are some neurons more vulnerable?

In cortical regions affected by AD, populations of neurons with specific laminar distribution and connectivity patterns are particularly susceptible to degeneration, whereas other neurons remain generally undamaged. This differential vulnerability has been described in terms of morphologic and biochemical characteristics of distinct neuronal populations. Pathology also follows regional hierarchies of severity: primary sensory cortices are less severely affected than related and higher-level association areas (Hof and Morrison 1994; Giannakopoulos et al. 1998; Braak et al. 2011). Selective vulnerability to form NFTs and/or DNs may be linked to the features of different subgroups of neurons: in particular, the type of efferent and afferent connections these neurons make, and the protein components that comprise their cytoskeleton (Vickers et al. 2000).

1.4.1 Cortico-cortical projection neurons are vulnerable in AD

Functional organization in the cortex is based on cellular characteristics and neural connections, of which there are four types. These are: projection fibres to subcortical regions (originating mostly in layers 5 & 6), callosal fibres to contralateral cortex, association fibres to ipsilateral cortex (collectively known as corticocortical fibres) and thalamocortical fibres which provide virtually all extrinsic input to the cortex, with the exception of the olfactory system (Douglas and Martin 2004). Corticocortical projections originate mostly in layers 2/3 and to some extent 5, and

can be classified as feed-forward, feed-back or lateral connections depending on their destination (Thomson and Bannister 2002; Fame et al. 2010). Feed-forward connections ascend within a hierarchically organized system (i.e. primary sensory area to association area), while feed-back connections descend the same hierarchy, and lateral projections connect cortical regions of the same functional level in separate hierarchies. Feed-forward connections usually originate from neurons located in the supragranular layers and terminate in layer 5 and/or the deep portion of layer 3 of the target region. Feed-back projections emanate from the deep layers (infragranular) and terminate in layers 1 and 6, while lateral connections arise from the deep layers and project to all layers of the target region (Douglas and Martin 2004).

The regional and laminar distribution of Aβ deposits (Duyckaerts et al. 1986) and NFTs (Lewis et al. 1987) in the AD neocortex suggests that the latter are closely associated with the cells of origin of corticocortical projections, whereas the distribution of plaques appears to correlate with the termination of such projections (Hof and Morrison 1990a,b; Vickers et al. 2000). The predominant localization of NFT pathology in layers 3 and 5 suggests that all types of corticocortical connections might be affected. In association cortical areas, NFTs predominate in layer 5 (Lewis et al. 1987), suggesting that feed-back and lateral projections are mostly affected. However, in primary and secondary sensory cortices, feed-forward projections are implicated as NFTs are more concentrated in superficial layers. Similarly, the preferential distribution of Aβ deposits in supragranular layers 2/3, as well as layer 4, the main target of efferent thalamocortical fibres, has prompted some to suggest that they reflect the degeneration of terminating corticocortical and thalamocortical projections, respectively (Duyckaerts et al. 1986). Corticofugal connections

emanating largely from layers 5 and 6, targeting subcortical structures such as the thalamus, striatum, brain stem and spinal cord, appear to be resistant to AD pathology (Hof et al. 1999; Vickers et al. 2000).

Well-defined projections in the hippocampal formation display a pattern of AD pathology distribution comparable to that of neocortical circuits (Hyman et al. 1984). The perforant pathway, arising from layer 2 in the entorhinal cortex (EC) and terminating in the dentate gyrus of the hippocampus, is consistently and severely affected early in AD (Gomez-Isla et al. 1996; Stranahan and Mattson 2010). Likewise, a high density of NFTs in the CA1 field and subiculum in the hippocampus parallels the degeneration of connections to specific nuclei in the amygdalae and other limbic or association cortical areas. For example, there is a high density of Aβ deposits in the mediobasal nucleus of the amygdala that receives projections from the EC, while EC layers 1 and 3 that receive afferents from the hippocampus and amygdala, have the highest Aβ plaque load in the EC (Hyman et al. 1990). Such specific lesion distribution in both the neocortex and archicortex reflects a hierarchy of connections that is differentially affected in AD.

Neuroimaging studies in human cohorts have also provided evidence for selective corticocortical fibre degeneration. Structural MRI studies of AD patients have revealed an early, pronounced commissural fibre loss and callosal atrophy, particularly in the temporal lobe (Thompson et al. 1998; Hampel et al. 2002; Teipel et al. 2002). Similarly, functional brain imaging studies indicate alterations of corticocortical connections, especially between prefrontal and medial temporal lobe regions (Grady et al. 2001; Brier et al. 2012). Dementia severity correlates well with the degree of callosal atrophy, suggesting that disconnections lead to functional deficits (Teipel et al. 2003). Loss or disruption of corticocortical connections is

specific to Aβ-rich areas and occurs even in asymptomatic patients with high Aβ loads, and may be indicative of local hypometabolism (Drzezga et al. 2011).

More recently, data from transgenic mouse models has highlighted the vulnerability of specific corticocortical networks. Retrograde tracing in APP/PS1 mice has shown that fibres originating from the cortex and terminating in both distal and proximal cortical areas contained numerous DNs, whereas those that originated subcortically showed infrequent disruptions (Delatour et al. 2004; Adalbert et al. 2009). These mice have also been shown to develop early (~2 months) commissural atrophy and loss of axonal densities, prior to Aβ plaque deposition, suggesting that corticocortical projections might be specifically lost (Chen et al. 2011b). In APP23 mice, commissural layer 3 pyramidal neurons in the frontal cortex were particularly vulnerable to Aβ plaque toxicity (Capetillo-Zarate et al. 2006). Interestingly, in the same study, no loss of non-pyramidal commissural neurons was observed (Capetillo-Zarate et al. 2006). The implication is that although connection patterns may be significant, they are not the sole determinant of vulnerability to degeneration in AD (discussed below). Even more intriguing, a recent study by Romito-DiGiacomo and colleagues (2007) showed that layer-specific differences in Aβ susceptibility are preserved *in vitro*. By harvesting embryos at various stages of development, they were able to isolate and culture migrating neurons that would eventually give rise to supra- or infragranular populations. They showed that immature supragranular populations displayed a much higher susceptibility to Aβ toxicity than infragranular ones, closely mimicking the pattern seen in AD. This would suggest that apart from the types of connections these neurons make (i.e. predominantly corticocortical) there must also be cell-intrinsic factors that confer vulnerability.

1.4.2 Basal cholinergic neurons are vulnerable in AD

Organization of cholinergic input to cortex

The two primary sources of acetylcholine (ACh) signaling in the human and rodent brain are subcortical projection neurons that make widespread connections to distal areas and local cholinergic interneurons of the cortex (Mesulam 1995; von Engelhardt et al. 2007). Cholinergic projections neurons that provide the primary cholinergic input to limbic, cortical and hippocampal structures are found in the magnocellular basal forebrain cholinergic system (CBF). This is comprised of the nucleus basalis magnocellularis (nBM, also referred to as nucleus basalis of Meynert), the horizontal and vertical limbs of the diagonal band of Broca (DB), and the medial septal (MS) nucleus (Woolf 1991; Newman et al. 2012). Specifically, cholinergic neurons in the MS predominantly innervate the hippocampus and entorhinal cortex (with some contribution from vertical limb DB neurons as well), whereas those of the vertical and horizontal DB mainly project into the anterior cingulate cortex and olfactory bulb, respectively (Mesulam 1995). The nBM provides most of the cholinergic input to the cortical mantle and the amygdala and forms diffuse synaptic connections throughout all layers of the neocortex, concentrating mostly in layers 2/3 and 4 (Kitt et al. 1994; Franklin and Paxinos 2008). The cholinergic system is strongly involved in modulating a wide range of cognitive domains including, working memory, attention, episodic memory encoding and spatial memory processing (reviewed in: Newman et al. 2012; Piciotto et al. 2012). Many of these cognitive functions become altered in both ageing and dementia, making research of the cholinergic signaling system of particular interest (Daulatazai 2010).

Targets of cholinergic input and types of receptors

ACh exerts its action by acting on nicotinic (nAChR) and muscarinic (mAChR) receptors which are located both pre- and post-synaptically (Piciotto et al. 2012).The former are ligand-gated ion channels with high cation permeability (Albuquerque et al., 2009), while the latter are metabotropic receptors that are either coupled to G_q proteins (M1, M3 and M5 subtypes) that activate phospholipase C or $G_{i/o}$ proteins (M2 and M4 subtypes) that negatively couple to adenylate cyclase (Jones et al. 2012) linking ACh neurotransmission to a variety of downstream signaling events. Nicotinic ACh receptors are non-selective, excitatory cation channels that induce depolarization and lead to Ca2+ influx upon activation. These receptors occur as homomeric or heteromeric complexes made up from a large family of α- and β-subunits (α2-α7 and β2-β4, reviewed in: Buckingham et al. 2009). Presynaptic nAChRs facilitate release of both inhibitory and excitatory neurotransmitters throughout the brain (Mckay et al. 2007). For example, activation of presynaptic α4β2 nAChRs on hippocampal interneurons causes release of GABA and inhibition of pyramidal neurons (Lena and Changeux 1997; Griguoli and Cherubini 2012). In contrast, activation of presynaptic α7 nAChRs on glutamatergic neurons enhances their neurotransmission (Dani and Bertrand 2007). α7 and α4β2 nAChRs can also be found post-synaptically where they are involved in plasticity mechanisms (Matsuyama et al. 2000). For example, the hippocampus is an important target where ACh can exert its modulatory effects on memory formation and learning (Kenney and Gould 2008). Consistent with this, the hippocampus has a very high density of nAChRs (Fabian-Fine et al. 2001) coupled with rich cholinergic innervation from the MS and DB (Frotscher and Lertanth 1985).

The 'Cholinergic Hypothesis' of AD

Given the important modulatory role that ACh plays in memory and learning, it was no surprise that a lot of early research into AD aetiology concentrated on this system. Indeed, early reports showed a profound loss (up to 70% in some studies) of CBF neurons in AD post-mortem cases (Bowen et al. 1976; Davies and Maloney 1976; Saper et al. 1985; Whitehouse et al. 1985). This was accompanied by a severe loss of cholinergic fibers in the temporal and frontal cortex (Geula and Mesulam 1996), as well as increased incidence of DN formation in cholinergic axons (Geula et al. 2008). Other studies reported significant reductions in the activity of two key enzymes in ACh metabolism: choline acetyltransferase (ChAT), the enzyme that synthesizes ACh, was found to be decreased in the neocortex (Slotkin et al. 1990; Ruberg et al. 1990; Lai et al. 2006) and locus cereleus (Strong et al. 1991; Lyness et al. 2003), while acetylcholine esterase (AChE), the main ACh degrading enzyme in the CNS and also widely used to label cholinergic axons, was also found to be decreased (Davies and Maloney 1976; Perry et al. 1978; Fishman et al. 1986; McGeer et al. 1986). The loss of CBF neurons and decreased production of ACh lead to the assumption that widespread cholinergic signalling deficits were the main cause of the cognitive symptoms observed in AD, a view espoused by the 'Cholinergic Hypothesis' of AD (Bartus et al. 1982). This in turn led to the approval of AChE inhibitors as a treatment for AD, with the aim to boost ACh availability and signalling (Di Santo et al. 2013). Although successful at targeting symptoms initially, these drugs do not address the underlying cause of the disease and soon lose their effectiveness.

However, more recent studies conducted in MCI and early-stage AD cases questioned the validity of the cholinergic hypothesis. In particular, the work by Davis

et al (1999) and DeKosky et al (2002) showed that ChAT activity was either preserved or even upregulated in some regions such as the hippocampus of MCI patients. Whether this was a pathological change or a compensatory sprouting response from septal cholinergic neurons following loss of hippocampal glutamatergic inputs remains unclear (Ikonomovic et al. 2003). Nevertheless, these studies corroborated previous reports of no change in AChE activity (Bierer et al. 1995; Shinotoh et al. 2000) or in ChAT-ir cell numbers (Rinnie et al. 1987; Gilmor et al. 1999) until moderate to severe stages of AD. Studies in Tg2576 (Apelt et al. 2002; Bednar et al. 2002; Wenk et al. 2004) and APP/PS1 (Hernandez et al. 2001; Machova et al. 2008) transgenic mice also failed to recapitulate CBF neuron loss and/or ChAT activity, albeit there were some reports of decreased cholinergic presynaptic terminals (Hu et al. 2003) and fibers (Luth et al. 2003).

Interestingly, there is evidence that many CBF neurons may actually shrink in size, persisting in this atrophied state as opposed to undergoing outright cell death (Pearson et al. 1983; Rinnie et al. 1987). CBF neurons were also shown to have deficits in growth factor signalling, likely leading to further dysfunction (Cuello et al. 2010). In particular, both the high (TrkA) and low (p75-NTR) affinity receptors for NGF were found to be down-regulated in CBF neurons in MCI and early-stage AD patients (Mufson et al. 2000; Chu et al. 2001; Mufson et al. 2002; Ginsberg et al. 2006a), likely affecting their signalling capacity. This means that loss of CBF neurons and/or axons was not the primary cause of cognitive decline in AD as previously stipulated (Bartus et al. 1982). Rather, it is the increasing dysfunction of CBF neurons that contributes to disease progression (Bartus 2000).

ACh receptors and Aβ– a toxic relationship?

Contributing to this sublethal dysfuntion of CBF neurons are more subtle changes occuring the ACh receptor level. Following the initial report that Aβ binds to α7-nAChRs with very high (picomolar) affinity, and to a lower extent α4β2-nAChRs (Wang et al. 2000), the interactions between Aβ and ACh receptors– both physiological and pathological– have been the subject of much research. Several groups, using different model systems, have now shown that nanomolar concentrations of $A\beta_{1-42}$ or $A\beta_{1-40}$ cause functional antagonism of both rat and human α7 nAChRs (Petit et al. 2001; Liu et al. 2001; Grassi et al. 2003; Wu et al. 2004; Pym et al. 2005), while picomolar concentrations activate α7 currents (Dineley et al. 2002; Dougherty et al. 2003; Wu et al. 2007; Puzzo et al. 2008; Parodi et al. 2010). Aβ effects on α4β2 are less clear, with some studies showing nanomolar (Wu et al. 2004) or micromolar (Lamb et al. 2005) concentrations eliciting receptor antagonism, while similar concentrations resulted in functional activation in other studies (Fu et al. 2003; Pym et al. 2005). In a more recent study, Wu et al (2008) showed that application of an α4β2 antagonist countered the effects of Aβ on LTP, while addition of ebatidine (an α4β2 agonist), potentiated them. Given that α4β2 are normally expressed presynaptically on inhibitory interneurons, activation of these receptors by Aβ would lead to increased firing and blockage of LTP (Jurgensen and Ferreira 2010).

However, studies employing subunit-specific antibodies showed marked decreases in immunoreactivity for α3 (Mousavi et al. 2003), α4 (Martin-Ruiz et al. 1999; Burghaus et al. 2000; Perry et al. 2000; Guan et al. 2000) and α7 (Banjaree et al. 2000; Wevers et al. 2000; Engidawork et al. 2001) subunits in AD tissue. Similarly, decreased binding of several subunit-specific radiolabelled ligands has also been

reported in post-mortem cases (Martin-Ruiz et al. 1999; Pimlott et al. 2004; Gotti et al. 2006; Lai et al. 2006; O'Brien et al. 2007), with loss of α4 and β2 binding the predominant trend. Losses in subunit expression (as determined by immunohistochemistry) were not accompanied by decreased in subunit mRNAs or decreases in neuron numbers, suggesting a post-translational defect such as aberrant sequestration and/or translation (Hellstrom-Lindahl et al. 1999; Wevers et al. 1999). This is furter supported by microarray data and RNA profiling from single neurons which show no changes in expression of nAChR genes (Ginsber et al. 2000; Wang et al. 2003; Yao et al. 2003). In contrast, less is known about the involvement of muscarinic receptors in AD. Contradictory findings of decreased (Nordberg et al. 1992) or unchanged (Mufson et al. 1998) levels of M2 receptors exist. Similarly, although levels of M1 receptors are reportedly unchanged (Svensson et al. 1992), more recent studies indicate that receptor coupling to downstream signalling pathways could be perturbed (Tsang et al. 2006).

Interestingly, non-neuronal cells seem to have elevated patterns of nAChR expression. For example, astrocytes in AD brains were found to have more intense immunolabelling and higher expression of α7 (Teaktong et al. 2004; Yu et al. 2005), while cultured rat astrocytes increased mRNA expression of all α4, α7 and β2 subunits (Xiu et al. 2005). More recently, Talantova et al (2013) showed that Aβ binds to astrocytic α7 receptors, eliciting a powerful relase of glutamate. Furthermore, neurons located close to Aβ deposits were found to have a normal complement of α4/α7 mRNA transcipts, unlike those with high NFT burdens (Wevers et al. 1999). This implies that Aβ interactions with α4- and/or α7-containing nAChRs could be an important process in AD pathogenesis. In this respect, increased nAChR signalling has been shown to lead to increased tau

phosphorylation *in vitro* (Wang et al. 2003), in 3xTG mice (Oddo et al. 2005), as well as in single CBF neurons in AD brains (Ginsberg et al. 2006b). Therefore, at early stages of AD, there could be an increase activation of nAChRs (probably α7 > α4β2), which in turn would cause increase in presynaptic facilitation (see below) and glutamate release from neurona and non-neuronal cells (Liu et al. 2013; Talantova et al. 2013). As the disease progresses, however, decreases in nAChR expression– either due to compensatory receptor internalization, or due to decreases in protein/mRNA levels– would then reduce the importance of this interaction in favour of other pathological processes such as neuronal death.

1.4.3 Neurons expressing neurofilament triplets are vulnerable in AD

As previously discussed, abnormally phosphorylated NFs accumulate early in DNs found in preclinical AD (Dickson et al. 1999; Woodhouse et al. 2009). Neurofilament triplet proteins belong to the type IV intermediate cytoskeletal filament family, and are predominantly expressed in a subpopulation of neurons deriving from pyramidal cells of cortical layers 2-6 that give rise to corticocortical connections (Vickers and Costa 1992; Hof et al. 1995; van der Gucht et al. 2007; Paulussen et al. 2011). The NF 'triplet' refers to the three genetically and structurally interrelated subunits [68 kDa (NF-L), 160 kDa (NF-M) and 200 kDa (NF-H)] that co-express and co-polymerize to form intermediate filaments in this subset of neurons (Vickers and Costa 1992; Lee and Cleveland 1996; Kirkcaldie et al. 2002; Lariviere and Julien 2003). In the rat neocortex, NF-immunopositive pyramidal neurons account for approximately 10-13% of all neurons (Kirkcaldie et al. 2002), whereas 20-30% of neurons in human temporal cortex are NF-immunopositive (Hof et al. 1990). The effect of AD on NF-containing neuronal populations has been studied as a possible insight to the basis of neuronal vulnerability. In macaques, the

vast majority (45-90%) of long association pathways interconnecting the frontal, temporal and parietal neocortex are NF-immunopositive, while short corticocortical, callosal and limbic pathways are characterized by lower numbers of such neurons (4-35%) (Hof et al. 1995).

Numerous studies employing the SMI32 antibody (recognizes dephosphorylated epitopes of the NF-M and NF-H subunits but does not cross-react with paired helical filaments, PHF-tau), indicate that a subset of layer 2, 3 and 5 pyramidal neurons containing NFs may be particularly susceptible to neurofibrillary pathology (Lewis et al. 1987; Morrison et al. 1987; Hof et al. 1990; Hof and Morrison 1990; Hof et al. 1995; Mann et al. 1996). NF triplet-containing neurons in superior frontal and inferior medial temporal association cortices (Hof et al. 1990), primary and secondary visual cortex (Hof and Morrison 1990) and hippocampal and entorhinal regions (Vickers et al. 1992, 1994) show a high degree of vulnerability for NFT formation and degeneration. Essentially, this vulnerable neuronal population is the myelinated subset of pyramidal neurons that give rise to specific corticocortical projections in the mammalian brain (Hof et al. 1995). Interestingly, cortical neurons that lack the NF-triplet, such as some inhibitory interneurons, do not develop NFTs and show a much lower susceptibility to degeneration in AD (Hof et al. 1991, 1993; Sampson et al. 1997; discussed below). Conversely, subpopulations of inhibitory neurons which do express the NF triplet are more susceptible to AD pathology and develop NFTs (Sampson et al. 1997). These findings provide evidence that a neuron's content of the NF-triplet correlates to its specific vulnerability to AD-like changes, and that these dephosphorylated NFs form focal accumulations that correspond to initial NFT formation that is distinct from tau-labelled NFTs (Vickers et al. 1992, 1994).

Studies have also demonstrated that specific NF-related changes occur in AD, such as abnormal, age-related phosphorylation of NF triplets in neuronal somata (labelled with SMI312 which recognizes phosphorylated epitopes of the NF-M and NF-H subunits; Masliah et al. 1993). Indeed, NFs found near Aβ plaques harbour multiple novel phosphorylation sites that are not normally expressed (Liao et al. 2004). This is accompanied by widespread increases in hyperphosphorylated forms of all three NF subunits in both AD brains (Wang et al. 2001) and mouse AD model tissue (Yang et al. 2009). Expression patterns of neurofilaments are also perturbed: there are reductions in NF-L mRNA levels (McLachlan et al. 1988) and immunoreactivity (Nakamura et al. 1997). There are also significant increases in the levels of NF-M and NF-H (Brettschneider et al. 2006), as well as NF-L (Pijnenburg et al. 2007) in the cerebrospinal fluid of AD patients, likely indicating increased axonal degeneration. Interestingly this is accompanied by increased intrathecal production of autoantibodies to NFs, suggesting immune responses may also be involved (Bartos et al. 2012).

It has also been demonstrated in AD cases that NFs are found within a subset of plaque-associated dystrophic neurites (DNs) distinct from those labelled with thioflavine S and tau (Masliah et al. 1993; Nakamura et al. 1997; Dickson et al. 1999). A subset of cortical pyramidal neurons in the supragranular layer of human cortex are α-internexin positive while NF-triplet negative, defining a distinct subpopulation (Dickson et al. 2005). Also a type IV intermediate filament, α-internexin is closely related to and associated with the NF-triplet (Yuan et al. 2006). Its expression is highest during development, decreasing in favour of NF-triplet expression with maturity (Leriviere and Julien 2003). α-Internexin immunopositive neurons become involved relatively later in AD-related changes and degeneration

than those that are NF-triplet positive (Dickson et al. 2005), suggesting a hierarchical neuronal vulnerability that depends on the type of intermediate filament protein expressed. For example, DNs immunopositive for triplet NFs appear early (Masliah et al. 1993; Nakamura et al. 1997; Dickson et al. 1999) while those that are uniquely α-internexin-immunoreactive become involved much later on (Dickson et al. 2005; Woodhouse et al. 2009).

Cumulatively, these findings suggest that NFs correlate with susceptibility to AD in the select subpopulation of neurons that express them, due to a predisposition to developing cellular changes that result in neurofibrillary pathology in AD. Recent advances in functional brain imaging techniques have also revealed certain brain networks ('hubs') that are particularly susceptible to AD pathology even in presymptomatic stages (Buckner et al. 2009; Brier et al. 2012). It would be interesting to determine if regional vulnerability in AD is conferred by network-intrinsic properties (i.e 'top-down'), the cell-autonomous features of their constituents (i.e. 'bottom-up'), or a combination of the two.

1.4.4 Interneuron vulnerability in AD

Types of interneurons in the mammalian cortex

Pyramidal neurons have relatively stereotyped anatomical, physiological and molecular properties, but inhibitory interneurons, which constitute approximately 20-30% of neocortical neurons, have vastly diverse morphological, physiological, molecular and synaptic characteristics (DeFelipe 1993, 1997; Cauli et al. 1997; Kawaguchi and Kubota 1997; Gupta et al. 2000; Fishell and Rudy 2011). Depending on their laminar distribution, subtypes of interneurons receive a different mixture of excitatory and inhibitory inputs (Xu and Callaway 2009). They also differ in their

complements of postsynaptic receptors, giving them characteristic activation properties (Blatow et al. 2005). Their dendritic arborizations differ widely, and so most current classifications distinguish interneurons on the basis of axonal morphology (Markram et al. 2004). Data from electrophysiological and immunohistochemical studies have revealed that interneurons with distinct morphologies usually target stereotypical pyramidal neuron compartments and thus contribute qualitatively distinct kinds of inhibition (Mel and Schiller 2004).

Specifically, basket cells typically envelop pyramidal somata and proximal dendrites (Freund 2003), chandelier cells give rise to numerous 'candlestick' axonal cartridges targeting the axon initial segment (Taniguchi et al. 2013), 'bitufted' and 'bipolar' cells synapse on spines of distal pyramidal dendrites or other interneurons (DeFelipe 1993, 1997), and finally, Martinotti cells predominantly send long axons up to layer 1 where they synapse on pyramidal apical dendrites (Ma et al. 2006). This allows the interneurons to modulate pyramidal neuron activity at various levels. GABAergic terminals on the axon initial segment are in the unique position to 'gate' action potential generation at the source. Likewise, perisomatic inhibition allows presynaptic interneurons to control the 'gain' of summed action potentials and therefore the action potential discharge of their target neuron (Mel and Schiller 2004; Jadi et al. 2012). This allows for the phasing and synchronization of neuronal activity in characteristic frequencies (e.g. gamma oscillations) (Cobb et al. 1995). Finally, dendritic-targeting interneurons influence dendritic processing and integration allowing them to 'filter' inputs from different sources and affect local spine plasticity (Kubota et al. 2007).

Interneuron classes can also be segregated according to differential expression of immunohistochemical markers with varying degrees of overlap (DeFelipe 1997; Markram et al. 2004). In this regard, the intracellular calcium-binding proteins parvalbumin (PV), calretinin (CR) and calbindin-D28K (CB) have been used widely in a variety of species and proven instrumental in categorizing subtypes of interneurons (and in some cases pyramidal neurons) that express them (DeFelipe 1993, 1997; Kawaguchi and Kubota 1997; Hof et al. 1999; Xu et al. 2010). It should be noted, however, that there is quite a lot of variability in expression patterns between cortical regions (Xu et al. 2010), as well as species (Hof et al. 1999; Fame et al. 2010). Therefore, caution should be exercised when comparisons between species are made. Nevertheless, in rats (Kawaguchi and Kubota, 1997) and mice (Matyas et al. 2004; Xu et al. 2010; Langer and Helmchen 2012), these three calcium-binding proteins (CaBPs) have been utilized to label functionally distinct groups of interneurons with minimal overlap.

Parvalbumin is expressed predominantly by basket cells, the largest subgroup of interneurons, spanning from layer 2/3 to 6 in mice (Xu et al. 2010) and layers 2-6 in humans (Hof et al. 1999b). Along with calbindin, it also labels chandelier cells, which are mostly found in layers 2/3 (Taniguchi et al. 2013). Calbindin also labels interneurons with a wide range of morphologies, including fusiform, bipolar, stellate and horizontal, while a subpopulation is co-labeled by calretinin (Park et al. 2002; Xu et al. 2010). Finally, calretinin-immunopositive interneurons mostly have cell bodies found between layers 2/3 and 4 in mice and layers 2 to 4 in humans (DeFelipe 1997), giving axonal projections with bipolar, or occasionally multipolar, morphology (Park et al. 2002; Caputi et al. 2009). Calretinin also labels a subgroup (~30%) of somatostatin-positive cells in the mouse neocortex (Xu et al. 2006). In

rodents, a substantial population of intrinsic cholinergic bipolar neurons in layer 2/3 are also co-labelled with calretinin (von Engelhardt et al. 2007). Interestingly, calretinin interneurons originate from a different developmental niche (caudal ganglionic eminence) than other interneurons (medial ganglionic eminence), likely reflecting the different inhibitory roles of the interneuron subclasses these markers label (Xu et al. 2004).

Interneuron pathology in AD

Early studies in post-mortem AD tissue disagree on the susceptibility of different cortical interneurons to AD pathology (summarized in Table 1.1). Reductions in the number and size of PV-positive interneurons (Arai et al. 1987), as well as cortical levels of parvalbumin protein (Inaguma et al. 1992) have been reported. Other studies reported no overt cell loss of PV cells near plaques, but observed dystrophic neurites and aberrant sprouts in the 'candlestick' terminals of chandelier cells (Ferrer et al. 1993b, c; Fonseca et al. 1993; Thangavel et al. 2008). Some reports also found a decrease in the number and size of CB-immunolabelled interneurons throughout the cortex (Ichimaya et al. 1988; Nishiyama et al. 1993; Greene et al. 2001), while others localized reductions specifically to infragranular layers (Hof and Morrison 1991). The vulnerability of calbindin-containing cells to AD, and non-AD dementias (Ferrer et al. 1993b) may depend on the degree of disease severity (Ferrer et al. 1993a). Neuropeptide-containing interneurons, particularly those containing somatostatin (SOM), also seem to be reduced in AD (reviewed by Burgos-Ramos et al. 2008; see Table 1.1).

Among all the other neuropeptides examined in post-morten AD tissue, SOM has consistently been implicated in AD pathogenesis. Firstly, there are reductions of more than 50% in SOM-ir interneuron numbers and/or their processes in the frontal

and temporal cortices of AD patients (Rossor et al. 1980b; Beal et al. 1986b; Bouras et al., 1986; Gaspar et al. 1989; Mazurek and Beal 1991) which correlate well with dementia severity (Dournaud et al. 1995). There are also reductions in the binding capacity of radiolabelled SOM-like ligands as well as decreases in SOM receptor subtypes 2, 3 and 5 immunolabelleling suggesting a decreased expression of receptors (Davies et al. 1980; Beal et al. 1985; Krantic et al. 1992; Kumar et al. 2005). Finally, several studies have shown lower concentrations of SOM peptides in the cortex (Bissette et al., 1998) and CSF (Davis et al. 1988; Molchan et al. 1993) of AD patients. Pronounced losses in SOM-ir interneuron numbers have also been shown in APP/PS1 transgenic mice quite early in disease progression, before changes in the cholinergic, glutamatergic or GABAergic systems are seen (Ramos et al. 2006).

Corticotropin releasing hormone (CRH) is an important factor involved in many cognitive processes including learning, addiction and most notably in modulating the stress response by acting on the hypothalamic-pituitary-adrenal axis (Maras and Baram 2012). Reductions in CRH tissue (Bisette et al. 1985; Whitehouse et al. 1987) and CSF (May et al. 1987; Davis et al. 1999) concentrations and concomitant increases in CRH receptor levels (DeSouza et al. 1986), as well as reductions in CRH-ir fibers and increases of CRH-ir fragments in fibrillar plaques (Powers et al. 1987; Strubble et al. 1987) have been described in both frontal and temporal cortex in AD patients. In addition, CRH levels may also correlate with overall neuropsychological assessment such that increased cognitive impairement was seen in patients with significantly decreased CRH levels (Pomara et al. 1989).

Likewise, neuropeptide Y (NPY), the expression of which varies quite strongly in the mouse neocortex but is predominantly in the infragranular layers (Xu et al. 2010),

has also been implicated in AD. Although the density of NPY-ir neurons in the temporal cortex was marginally lower in AD brains compared to controls, the degree of fiber branching was greatly reduced and there were many distorted NPY immunopositive neurites, consistent with previous reports of NPY in dystrophic neurites of the hippocampus (Chan-Palay et al. 1986; Kowall and Beal 1988). As there is a large overlap between NPY-ir and SOM-ir interneurons in the cortex, the loss of NPY could reflect the loss of SOM-ir interneurons (Beal et al. 1986a; Ramos et al. 2006). Nevertheless, concentrations of NPY are reportedly lower in CSF and plasma from AD patients as well (Alom et al. 1990; Koide et al. 1995; Minthon et al. 1997).

NPY is involved in numerous functions such as food intake, learning and memory, mood and has been found to have a neuroprotective role in a variety of neurodegenerative conditions (Malva et al. 2012). Indeed, studies in transgenic AD mice have shown that addition of exogenous NPY can have a neuroprotevtive effect by reversing depressive-like behaviour and ameliorating some cognitive deficits (Rose et al., 2009; dos Santos 2013). Interestingly, Diez et al. (2000, 2003) found that in very old PDAPP or APP23 mice (~23 month old) the number of NPY-ir neurons was actually increased, potentially due to its neuroprotective roles, particularly in the hippocampus. In these same studies, the numbers of CCK-ir interneurons in the cortex were significantly decreased, consistent with preivous reports in human AD post-mortem tissue (Struble et al. 1987; Mazurek and Beal 1991; Diez et al. 2000, 2003). Another study, however, found no significant changes in overall cortical levels of CCK in 5 different regions in AD brains compared to controls (Ferrier et al., 1983). One potential reason for such discrepancies is the large number of post-translational modifications that CCK undergoes and the existence of

many differently-spliced forms both peripherally and in the CNS, liekely resulting in different antibody specificities depending on the tissue/region examined (Beinfeld et al. 2009). Neverthelesss, given the many important functions that neuropeptides modulate in the brain such as mood and axiety, it is highly likely that any loss and/or dysfunction of the peptidergic systems would have far-reaching effects.

Table 1.1 Neocortical GABAergic neuronal subpopulations in AD.

Marker	Region	Outcome	Reference
PV	FC, TC	Decrease in cell number and size	Arai et al. 1987
	PFC, ITC	No change in cell size or density	Hof et al. 1991
	TC	No change in cell density	Ferrer et al. 1991
	FC, PC, OC, TC	PV concentration lower in parahippocampal gyrus only	Inaguma et al. 1992
	TC	No change in cell density, some PV+ dystrophic neurites	Ferrer et al. 1993c
	TC	No loss in chandelier cell density, however 30-35% decrease in candlesticks in layers 2-3	Fonseca et al. 1993
	SFC	PV+/NF– cells don't contain NFTs, however PV+/NF+ cells contain some NFTs	Sampson et al. 1997
	VC	No change in cell density, however smaller cell size	Leuba et al. 1998
	PPHG	No change in cell density	Thangavel et al. 2008
CR	PFC, ITC	No change in cell density/morphology	Hof et al. 1993
	TC	No change in cell morphology/distribution	Fonseca & Soriano 1995
	SFC	CR+ cells don't contain NFTs, more CR+ cells in layer 2	Sampson et al. 1997
	VC	No change in cell density, however smaller cell size	Leuba et al. 1998
CB	FC, TC, PC	Decrease in cell number and size	Ichimaya et al. 1988
	PFC	Layer 2-3 CB+ cells unaffected, layer 5-6 CB+ cells decreased. Layer 3 CB+ pyramidal neurons decreased	Hof & Morrison 1991

	TC	Decrease in cell number and number of dendritic branches, but only in severe AD cases	Ferrer et al. 1993a
	FC, TC	Layer 2 CB+ lost in AD and also age-dependently in controls	Nishiyama et al. 1993
	TC	No change in CB mRNA	Maguire-Zeiss et al. 1995
	VC	No change in cell density	Leuba et al. 1998
	TC	Up to 80% decrease in CB+ neurons	Greene et al. 2001
NPs	TC	Decrease in SOM radio-immunolabelling	Davies et al. 1980
	TC	47% decrease in SOM immunoreactivity	Rossor et al. 1980b
	TC	No difference in VIP immunoreactivity	Rossor et al. 1980a
	TC	No difference in CCK immunoreactivity	Rossor et al. 1981
	TC, PC, FC	Widespread reductions in SOM+ cells/processes	Bouras et al. 1986
	FC	60-80% decrease in SOM+ cell density (layers 2-3 & 5); no change in NPY	Gaspar et al. 1989
	FC, TC	CCK+ density reduced by 24-38% SOM+ density reduced by 45-65%	Mazurek & Beal 1991

TC temporal cortex, *FC* frontal cortex, *PFC* prefrontal cortex, *ITC* inferior temporal cortex, *OC* occipital cortex, *PC* parietal cortex, *VC* visual cortex, *SFC* superior frontal cortex, *PPHG* posterior parahippocampal gyrus, *PV* parvalbumin, *CR* calretinin, *CB* calbindin, *NPs* neuropeptides, *SOM* somatostatin, *VIP* vascular intestinal peptide, *NPY* neuropeptide Y, *CCK* cholecystokinin

Interneuron populations have also been studied in post-mortem AD human hippocampal tissue (Table 1.2). Like neocortex, decreases in the number of PV-positive interneurons have been reported throughout the hippocampus (in DG, CA1-2; Brady and Mufson 1997) and entorhinal cortex (Solodkin et al. 1996; Mikkonen et al. 1997). Likewise, the density of CB-immunolabelled interneurons is significantly reduced in CA fields 1-2 (Maguire-Zeiss et al. 1995; Iritani et al. 2001) and layer 2 of the entorhinal cortex (Mikkonen et al. 1997; Thorns et al. 2001). Interestingly, a

recent study by Koliastos and colleagues (2006) reported a sharp increase in the number of small CB-positive cells that also labelled for NADPH/nNOS (nitrergic neurons), particularly in the vicinity of Aβ plaques or NFT-bearing neurons. This higher expression of nNOS also correlated with levels of activated caspase-3, a well-known marker of apoptosis, suggesting that these cells may be involved in local cell death signalling (Koliastos et al. 2006). While most CR-immunoreactive cells in the hippocampal formation appeared to be preserved (Mikkonen et al. 1999), some have reported CR+ DNs associated with Aβ plaques in the subiculum and parahippocampal gyrus (Brion and Resibois 1994).

Table 1.2 Hippocampal GABAergic neuronal subpopulations in AD.

Marker	Region	Outcome	Reference
PV	EC	Density decreased, particularly layer 2	Solodkin et al. 1996
	Hipp	Up to 60% decrease in density in DG and CA1-2; no change in CA3, subiculum or presubiculum	Brady & Mufson 1997
	EC	Density decreased	Mikkonen et al. 1999
CR	Hipp	Some CR+ dystrophic neurites (subiculum & parahippocampal gyrus)	Brion & Resibois 1994
	EC	Density preserved	Mikkonen et al. 1999
CB	Hipp	Decreased CB+ staining in CA2	Maguire-Zeiss et al. 1995
	EC	Density decreased, CB+ pyramidal neurons preserved	Mikkonen et al. 1999
	Hipp	CB+ pyramidal neurons reduced in CA1-2 only in early onset AD	Iritani et al. 2001
	EC	Reduced density in layer 2	Thorns et al. 2001
	EC, Hipp	Increased density of CB+/NADPHd/nNOS+ interneurons in areas with high NFTs; correlate with activated caspase-3	Koliastos et al. 2006

EC entorhinal cortex, *Hipp* hippocampus

In contrast to PV+ and CB+ interneurons, numerous reports in AD cortex (Hof et al. 1993; Fonseca and Soriano 1995; Sampson et al. 1997; Leuba et al. 1998) and hippocampus (Mikkonen et al. 1999), as well as a canine model of dementia (Pugliese et al. 2004), have demonstrated that CR-immunolabelled cells display a relative resistance to AD-related pathology. Moreover, CR expression does not seem to be affected (compared to CB and PV) in a variety of other neurological disorders including schizophrenia (Hashimoto et al. 2003), multiple sclerosis (Clements et al. 2008) and epilepsy (Aronica et al. 2007). It is unclear what factors mediate such differential vulnerability in distinct interneuron populations.

Calretinin, like parvalbumin and calbindin-D28K, plays a critical role in modulating intrinsic neuronal excitability and plasticity via its calcium-buffering qualities (Camp and Wijesinghe 2009). Initially it was assumed that the relative resistance of interneurons to AD pathology (compared to pyramidal neurons) was due to their natural ability to buffer the calcium-mediated effects of excitotoxic stimuli (Hof et al., 1991; Hof and Morrison, 1992). In this regard, Greene and colleagues (2001) suggest that calcium-buffering proteins like calbindin are upregulated in some pyramidal cell populations during normal ageing, as a potentially neuroprotective mechanism. Any depletion in AD could render the host pyramidal neuron vulnerable to excitotoxic changes mediated by Aβ (Greene et al. 2001). For example, age-related decreases in calbindin could account for the selective loss of basal forebrain cholinergic neurons in AD, as the neurons containing high levels of calbindin did not develop neurofibrillary tangles or degenerate (Riascos et al. 2011). The three proteins have different Ca^{2+}-binding capacities, so differences in neurons' relative susceptibility to AD could be attributed to the buffering capacity of their calcium binding proteins (Schwaller 2009). Therefore, more research is needed to indicate the

extent to which interneurons are able to withstand excitotoxic insults *in vivo*, and whether this depends on the particular calcium binding proteins they express.

Another intriguing possibility is that subtypes of interneurons could vary in their response to Aβ toxicity depending on the type of cytoskeletal proteins they express. In the human cerebral cortex, the vast majority of CR-immunopositive cells do not contain the neurofilament triplet proteins (Hof et al. 1990; Hof and Morrison 1990; Hof et al. 1999). A very small subpopulation of large, horizontal calretinin cells that in the human brain may correspond to Cajal-Retzius cells in cortical layer 1 (Fonseca and Soriano 1995), has been previously found to weakly co-label with the NF marker SMI32 (Sampson et al. 1997). Interestingly, this small population of NF-containing calretinin-positive interneurons undergoes cytoskeletal alterations and neurofibrillary pathology in AD, resulting in a more than 50% cell loss (Sampson et al. 1997). Additionally, there are subpopulations of PV- and CB-positive interneurons that also express NFs, which are also highly susceptible to AD pathology (Hof et al. 1993; Sampson et al. 1997). These findings are in agreement with the general hypothesis that NF (triplet) -containing neurons, regardless of their lineage, are predisposed to AD-related pathology (Vickers et al. 1996, 1997, 2000). Conversely, neurons in the cortex that lack the NF triplet proteins may contain other neuronal intermediate filament proteins, such as α-internexin, and be less susceptible to AD pathology (Dickson et al. 2005).

1.5 Synaptic pathology in AD

The physical connection framework of the brain is widely considered to be the substrate of memory and learning processes with the synapse as its fundamental unit. Synaptic dysfunction– ranging from altered synaptic dynamics and plasticity

processes, to full-blown synapse loss– is the earliest pathological feature of AD (Huang and Mucke 2012; Sheng et al. 2012). It likely begins years before functional deficits become apparent, during the incipient preclinical phase of AD, and is widely believed to be mediated by the accumulation of toxic Aβ species (Benilova et al. 2012). As with other types of AD pathology, distinct types of synapses seem to be preferentially affected.

1.5.1 Synapse loss in AD

Since the first report of a significant correlation between synaptic loss and dementia severity (Davies et al. 1987), the effect of dementing diseases on synaptic proteins has been a subject of wide research. In AD, synapse dysfunction has been reported to occur very early in affected regions such as frontal or temporal cortex, and may correlate more closely with cognitive decline than Aβ plaques or neurofibrillary tangles (DeKosky and Scheff 1990; Terry et al. 1991; Scheff and Price 2006, 2007). Levels of numerous synaptic proteins, a useful measure of synaptic integrity, are reduced in both human AD cases (Honer et al. 2003) and virtually all transgenic AD mouse models (Crews et al. 2010). However, the degree to which synaptic proteins change in AD is synapse-type and brain-region specific; distinct neurotransmitter systems also appear to be differentially affected by AD progression (Gsell 2004). In this regard, cholinergic transmission seems to be affected very early, while GABAergic signalling appears to be relatively preserved even in late AD (Davies et al. 1998). Investigating the synaptic targets affected by AD pathology, as well as the mechanisms by which this synaptotoxicity is mediated, would be highly informative and may provide novel therapeutic targets.

Although plaque load measurements in post-mortem tissue are not necessarily correlated with the rate of cognitive decline in AD (Braak et al. 2011), it is

interesting that synapse loss seems to be accentuated within or near fibrillar Aβ plaques in both human AD cases (Masliah et al. 1994; Dessi et al. 1997; Samuel et al. 1997; Sze et al. 1997) and many transgenic mouse models (Bell et al. 2003, 2006; Hu et al. 2003; Dong et al. 2007; Koffie et al. 2009), but is not exacerbated by diffuse Aβ deposits (Masliah et al. 1990). Moreover, recent studies utilizing brain imaging probes for fibrillar Aβ such as [^{11}C]PiB-PET (Edison et al. 2007), as well as plasma (Cosentino et al. 2010) and cerebrospinal fluid levels of soluble $A\beta_{1-42}$ (Santos et al. 2012), have yielded promising correlations with disease severity. This evidence is suggestive that Aβ, either in its toxic soluble oligomer conformation (Hardy and Selkoe, 2002) or as insoluble fibrillar deposits (Dong et al. 2007), could play a driving role in synapse loss in AD. A recent study showing Aβ plaques acting as dynamic sources of soluble oligomers *in vivo*, suggests that the distinction may be unimportant in the brain anyway (Koffie et al. 2009). More importantly, the site of Aβ toxicity (pre- or post-synaptic), as well as the specific mechanism(s), need to be better defined in order to yield potential therapeutic targets.

1.5.2 Presynaptic effects of Aβ

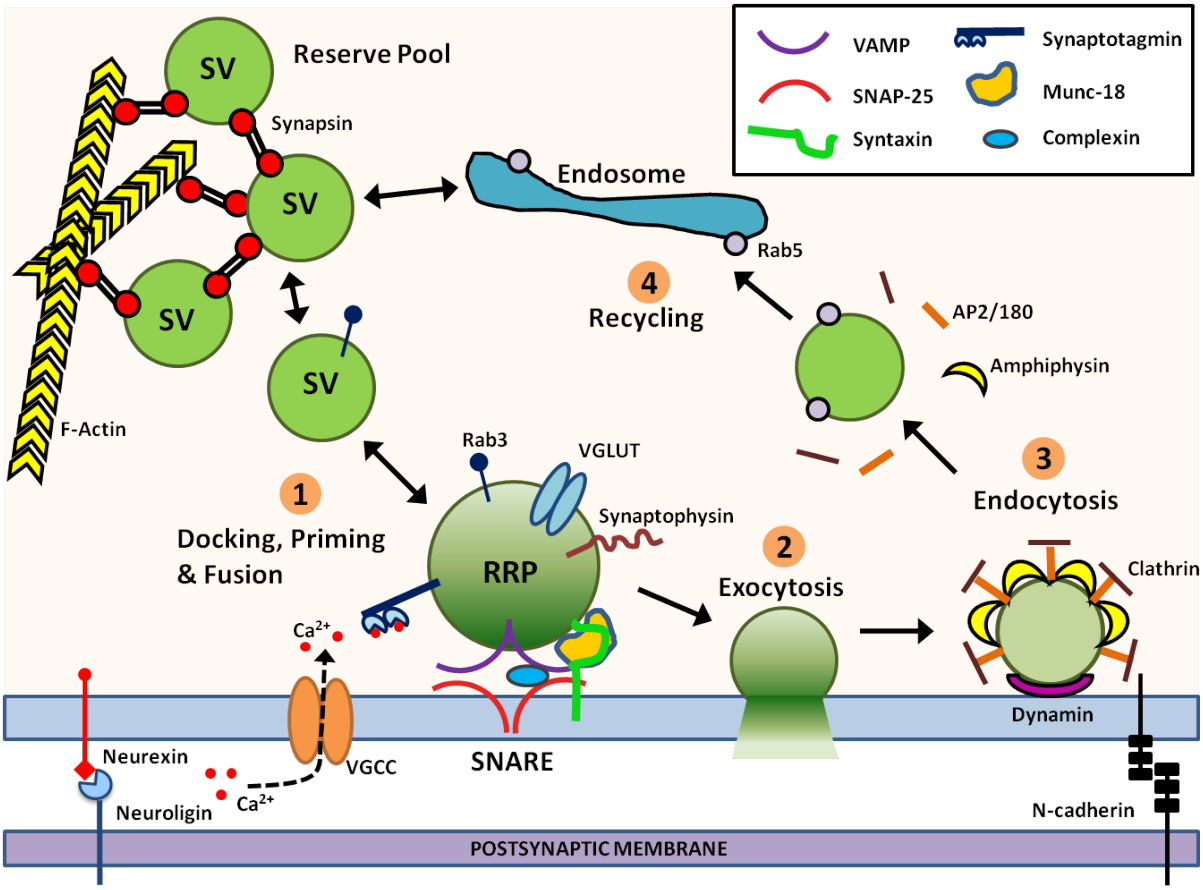
Presynaptic pathology was first implicated in AD by early immunohistochemical post-mortem studies that showed reductions in the ubiquitous presynaptic marker, synaptophysin (Masliah et al. 1990, 1993, 1994; Terry et al. 1991). Since then many studies have identified alterations of presynaptic function, and loss of presynaptic terminals, as early targets of Aβ toxicity that could contribute significantly to initial functional deficits in AD. Many of these have focused on proteins integral to the synaptic vesicle (SV) cycle, the pathway by which neurons: i) package neurotransmitters into SVs and transport them to the cell membrane for exocytosis and chemical signalling, ii) endocytose neurotransmitters from the synaptic cleft and

recycle SV membranes, and iii) maintain the resting SV pool. These processes have received particular attention because Aβ is secreted by presynaptic terminals, where its precursor APP is actively transported (Buxbaum et al. 1998; Lazarov et al. 2002). Alterations of presynaptic proteins involved in the SV cycle and pre- to post-synaptic adhesion have been reported in human AD cases and TG mice.

Trafficking/Exocytosis-related proteins

The primary function of presynaptic boutons is to facilitate the controlled, rapid release of neurotransmitter from SVs. This is achieved by exocytosis, which can be modulated by short- and long-term plasticity mechanisms that regulate the number and release probability of SVs that participate. The ability of individual boutons to dynamically modulate the rate and amount of neurotransmitter release is an essential requirement for proper brain function. SV exocytosis involves three critical steps (docking, priming and fusion) and occurs at highly specialized regions of the presynaptic membrane called active zones (AZs) (Figure 1.1; Sudhof 2012). These AZs are composed of a series of multi-domain proteins, including structural elements and Rab3a-interacting molecules, Muncs, Piccolo and Bassoon that collectively function as organizers or modulators of neurotransmitter release (Figure 1.1; Sigrist and Schmitz 2011).

Figure 1.1 Structural elements of the presynaptic active zone. (adapted from Waites and Garner 2011).



Before SVs can reach the AZ and become primed for release, they first need to be recruited from the reserve pool. The small G-protein member of the Rab family, Rab3A, critically regulates SV recruitment (Leenders et al. 2001), and along with Munc-18, docks them to the plasma membrane where they can be primed (Dulubova et al. 2005). Priming prepares SVs for rapid fusion in response to Ca^{2+} influx and is facilitated by Munc-18 binding syntaxin-1a to initiate fusion by assembling the SNARE complex (soluble *N*-ethylmaleimide sensitive factor attachment protein (SNAP) receptor) (Smyth et al. 2010). SNARE comprises three proteins: vesicle-associated membrane protein (VAMP)-2/synaptobrevin (on the SV), and SNAP-25 and syntaxin-1a (on the plasma membrane) (Parpura and Mohideen 2008). Fusion is initiated when synaptotagmin triggers an increase in SV membrane curvature in

response to action potential mediated Ca^{2+} influx (Fernandez-Chacon et al. 2001; Martens et al. 2007).

Fusion is tightly regulated by complexins, which bind the SNARE complex to inhibit (Yang et al. 2010) or facilitate (Xue et al. 2010), depending on the level of intracellular Ca^{2+}. Loss of complexin interaction with the SNARE complex has also been shown to result in increased levels of spontaneous, asynchronous SV fusion and neurotransmitter release (Yang et al. 2010), indicating that complexins are crucial for coordinating exocytosis. SNARE assembly is also regulated by the synaptophysin, which binds to synaptobrevin and restricts its availability to interact with the other SNAREs (Pennuto et al. 2002). Although it is the most abundant (> 10%) SV protein (Takamori et al. 2006), knock-out studies show that it is not necessary for neurotransmitter release (McMahon et al. 1996). Instead, it has also been implicated in SV endocytosis via recruitment of dynamin (Daly and Ziff 2002), a critical regulator of the SV endocytotic pathway (see below).

Numerous studies support a role for the SNARE complex and associated proteins in affected brain regions in AD. Studies of post-mortem tissue have reported significant decreases in mRNA, protein and immunohistochemical intensity of synaptobrevin (Ferrer et al. 1998; Sze et al. 2000; Honer et al. 2012), SNAP-25 (Yoo et al. 2001; Beeri et al. 2012) and syntaxin-1a (Minger et al. 2001; Love et al. 2006; Pham et al. 2010) at early stages of AD. Similarly, synaptotagmin (Davidsson and Blennow 1998; Sze et al. 2000; Yoo et al. 2001; Reddy et al. 2005) and complexin mRNA and protein levels are also decreased at initial stages of AD. Impaired SNARE machinery in AD could lead to a decreased capacity for SV docking and fusion and reduced signalling. Conversely, loss in SV fusion regulators such as complexin may dysregulate spontaneous SV fusion causing aberrant release of neurotransmitters.

Other AZ proteins also show potentially pathological alterations in AD. Sustained Rab3a mRNA down-regulation is an early feature in AD progression (Reddy et al. 2005; Emilsson et al. 2006; Saetre et al. 2011) while Rab3a protein levels (Sze et al. 2000) and immunoreactivity (Davidsson and Blenow 1998) are decreased, perhaps leading to reduced recruitment from the reserve pool and altered docking dynamics. Interestingly, Munc18 is upregulated (Jacobs et al. 2006) and aberrantly hyperphosphorylated in AD tissue, potentially altering its binding and interaction with Rab3a and syntaxin-1a (Park et al. 2012). Whether this is compensation for declining levels of SNARE proteins and Rab3, or a byproduct of Aβ toxicity, is not yet clear. Recently, Aβ has also been shown to disrupt synaptophysin binding to synaptobrevin (Russel et al. 2012) *in vitro*, facilitating synaptobrevin participation in exocytosis and enhancing SV release. This could be further potentiated *in vivo* by the early pronounced loss of synaptophysin in AD (Terry et al. 1991). As AD progresses, overall synaptophysin reduction correlates well with cognitive symptoms (Masliah et al. 1992), NFT counts (Lassman et al. 1992; Callahan et al. 2002), and Aβ plaque load (Sze et al. 1997; Lue et al. 1999).

Endocytosis/Vesicle recycling-related proteins

Maintenance of SV pools during sustained activity requires the rapid recycling of SVs after exocytosis, including the retrieval of structural proteins and membranes by endocytosis. Most SV endocytosis is accomplished by clathrin, a molecular triskelion that assembles into a lattice on vesicle membranes (Figure 1.1, Granseth et al. 2006). Recruitment of clathrin to the membrane of newly invaginating vesicles (clathrin-coated pits) is mediated by a family of adaptor proteins (APs), notably AP-2 (Voglmeier et al. 2006) and AP-180 (Koo et al. 2011). APs also help to concentrate and sort cargo proteins destined for endocytosis and play a crucial role in

determining SV size and quanta, as knock-down of either protein usually results in fewer, enlarged vesicles (Zhang et al. 1998; Meyerholz et al. 2005). As the new vesicle forms, amphiphysin1 promotes membrane curvature and recruits dynamin to the complex (Wu et al. 2009), enabling scission from the plasma membrane (Roux et al. 2006).

There is now ample evidence that early-endosomal dysfunction is a common pathological mechanism in AD that contributes to presynaptic deterioration, and also directly influences APP metabolism and Aβ production (Nixon 2005). AD tissue shows marked reductions in key endocytosis proteins, specifically AP-2 (Yao and Coleman 1998b), AP-3 (Yao and Coleman 1998a), AP-180 (Yao et al. 2003), amphiphysin-1 (De Jesus-Cortes et al. 2012) and dynamin 1 (Yao et al. 2003; Tannenberg et al. 2006). This is consistent with observed depletion of dynamin 1 in the brains of TG mice (Kelly et al. 2005) and neuronal cultures following Aβ treatment (Watanabe et al. 2010). Such reductions in active dynamin protein could result in a reduced capacity for SV recycling and therefore a diminished SV pool (Kelly and Ferreira 2007).

More recent studies, however, report increased dynamin 1 and 2 expression in young Tg2576 mice (Thomas et al. 2011) accompanying a spike in Aβ production, and that *in vitro* ablation of either dynamin gene results in reduction of Aβ levels (Yu et al. 2010). This is most likely because APP endocytosis is critical for Aβ generation in early-endosomes (Koo and Squazzo 1994); since APP internalization and Aβ production are clathrin-mediated (Cirrito et al. 2008), there is probably a role for the dynamin GTPases in Aβ metabolism. Dynamin 1 has been shown to further bolster Aβ generation by influencing the endosomal localization of BACE1, required for amyloidogenic cleavage of APP (Zhu et al. 2012). The contradiction between the

global depletion of dynamin in AD, and dynamins' pivotal role in Aβ metabolism, is intriguing and needs further research. One potential explanation is that this pathway could be downregulated by ageing neurons to compensate for increased Aβ load.

Recently, another key player in clathrin-mediated endocytosis has also been implicated in APP internalization and Aβ production. SNPs in the PICALM gene (phosphatidylinositol clathrin assembly lymphoid-myeloid leukemia, a ubiquitously expressed homologue of AP-180) have recently been identified as risk genes for late onset AD (Harold et al. 2009). Like AP-180, PICALM is a cytoplasmic adaptor protein localized at presynaptic boutons (Yao et al. 2005) where it plays a critical role in clathrin-mediated processes: binding AP-2 to form clathrin-coated pits for endocytosis (Tebar et al. 1999; Meyerholz et al. 2005) and SV vesicle fusion with early-endosomes and subsequent trafficking (Miller et al. 2011); knock-down of PICALM mislocalises synaptobrevin (Koo et al. 2011) further indicating a critical role in SV recycling. Disruption of PICALM/AP-180 function can dysregulate vesicle size (Zhang et al. 1998) possibly due to decreased synaptobrevin-mediated SV curvature (Martens et al. 2007). SV size is remarkably conserved across species at 35-42 nm (Takamori et al. 2006), possibly for energy efficiency (Lee and Schick 2008). Therefore, aberrant increases in SV size due to loss of AP-2 or AP-180 in AD could result in higher energy demands at the AZ.

Studies in large AD cohorts remain inconclusive on the effect of PICALM SNPs on Aβ production – some claim it increases cortical Aβ deposition (Schjeide et al. 2011), while others show no effect (Kauwe et al. 2011). In TG mice overexpressing mutant hAPP, PICALM is dose-dependently upregulated and co-localizes with Aβ in early-endosomes, but knock-down of PICALM prevents APP internalization and radically reduces Aβ production (Xiao et al. 2012). This suggests that, along with

dynamin 1, PICALM is critically involved in amyloidogenic APP processing in addition to clathrin-mediated SV endocytosis. This warrants further research, particularly in PICALM SNP-bearing human AD cases.

Although most SVs are reconstituted from the presynaptic membrane via clathrin-mediated endocytosis, SV recycling through endosomal intermediates is sometimes used. For instance, intense firing may trigger bulk endocytosis (Clayton and Cousin 2009), to make large presynaptic endosomes (Voglmeier et al. 2006) from which AP-3 mediates microvesicle formation (Blumstein et al. 2001). The GTPase Rab5 (Figure 1.1) is also critical for early-endosome formation and SV fusion with endosomes where cargo can be sorted and trafficked (Nixon et al. 2005). In AD, AP-3 mRNA and protein decreases, suggesting that more than one vesicle trafficking pathway is affected (Yao et al. 2003). Rab5-positive endosomes appear before other AD pathology (Cataldo et al. 2000), whereas late-endosomal components like Rab7 may be downregulated as disease progresses (Yuyama and Yanagisawa 2009). This would favour the accumulation of enlarged early-endosomal structures with increased APP metabolism and Aβ production (Vetrivel et al. 2006), while perturbing normal endosomal trafficking and SV recycling.

Maintenance of SV pools

Although SVs appear morphologically uniform, three functionally distinct pools are released under different circumstances: readily releasable SVs (~1%) are docked and primed at the AZ and fuse immediately upon Ca^{2+} influx; the recycling pool (10-15%), released during sustained stimulation when they continuously cycle through exo/endocytosis; and the reserve pool (80-90%), only released under very intense stimulation (see Rizzoli and Betz 2005). The distinction between these pools is

maintained primarily by synapsin (Cesca et al. 2010) and F-actin (Cingolani and Goda 2008; see below). Synapsins tether SVs to the actin cytoskeleton (Figure 1.1), but dissociate after activity-dependent phosphorylation, allowing SVs to translocate to the AZ for exocytosis (Chi et al. 2003). Knock-down of synapsin or depolymerisation of F-actin results in aberrant admixing of the reserve pool with the readily releasable pool, significantly altering the kinetics of neurotransmitter release (Jensen et al. 2007). Importantly, several synapsin sites are phosphorylated by multiple kinases, allowing fine-tuning of release kinetics to stimulus intensity (Cesca et al. 2010).

Pronounced decreases in mRNA expression and protein levels of both synapsin 1 (Qin et al. 2004) and synapsin 2 are observed in human AD (Ho et al. 2001; Yao et al. 2003) and in TG mice over-expressing mutant hAPP (Dickey et al. 2003; Pham et al. 2010). Presumably, loss of synapsins' tethering function would release more SVs from the reserve pool to join the readily releasable pool at the AZ; even physiological Aβ levels can result in an increase in the number of vesicles available at the presynaptic AZ via α7-subunit nicotinic acetylcholine receptor-mediated activation of downstream signalling cascades (Puzzo et al. 2008, 2011; Abramov et al. 2009). Chronic over-recruitment of vesicles, combined with a reduction in synapsin levels, would eventually result in severely depleted SV pools and compromised neurotransmission (Parodi et al. 2010).

Transporter and channel proteins

A key determinant of signalling strength is the content of neurotransmitter (quantal size) in SVs at the AZ. The quantal size is determined both by the local abundance of neurotransmitter in the cytoplasm as well as the number of vesicular transporters

available on the cytoplasmic surface of SVs (Wilson et al. 2005). These transporters load SVs with specific neurotransmitters: glutamate (VGlut-1/2/3; Fremeau et al. 2001), acetylcholine (VAChT; Weishe et al. 1996), monoamines (VMAT-1 & 2; Peter et al. 1995) or γ-aminobutyric acid (VGAT; McIntire et al. 1997).

On the supply side, special transporters exist on neuronal and glial cell membranes that actively re-uptake neurotransmitters from the extracellular space into the cytoplasm, to avoid desensitizing post-synaptic receptors by prolonged exposure to neurotransmitters. The best-characterized are the five Na^+ electrogradient-dependent excitatory amino acid transporters (EAATs). EAAT1 (or GLAST) and EAAT2 (or GLT-1) are found on glial and endothelial cells (Anderson and Swanson 2000), while EAAT3 (or EAAC1) and EAAT4 are found on principal neurons (Beart and O'Shea 2007); EAAT5 is predominantly found on bipolar neurons in the retina. Glutamate taken up by astrocytes via GLAST and GLT-1 is converted to glutamine, delivered to neurons, converted back to glutamate and loaded into SVs by VGluts (Eid et al. 2012). Collectively, this is known as the glutamate-glutamine cycle. The astroglial transporters, notably GLT-1 and variants, are responsible for around 90% of extracellular glutamate reuptake (Allaman et al. 2011).

AD processes interact with many of these elements. Glutamatergic synapse loading appears to be vulnerable: Aβ appears to accumulate in human VGlut-1 positive synaptosomes (Sokolow et al. 2012) and decreases in cortical VGlut-1 have been shown in end-stage human AD (Kirvell et al. 2006; Chen et al. 2011), correlating with cognitive symptoms (Kashani et al. 2008). Studies in TG mice over-expressing mutant hAPP also show reduced glutamatergic presynaptic boutons and VGlut-1 protein levels in Aβ plaque-rich regions (Bell et al. 2003, 2006; Cassano et al. 2012). This would reduce glutamate availability for filling SVs, decreasing glutamate

release at excitatory synapses (Minkeviciene et al. 2008); studies report reduced glutamate concentrations in human AD (Rupsingh et al. 2011) and TG mice (Cassano et al 2012; Hartmann et al. 2012; 3xTg and APP/PS1, respectively), supporting an early and pronounced loss of glutamatergic synapses during AD progression (Terry et al. 1991; Gsell et al. 2004; Bell et al. 2006).

AD also exhibits marked decreases in glutamate reuptake (Masliah et al. 1996; Liang et al. 2002) and reduced, aberrant expression of EAATs 1-3 (Li et al. 1997; Chen et al. 2011; Scott et al. 2002; Thai 2002; Duerson et al. 2009). Similar findings have been replicated in animal studies. Synaptosomes isolated from Sprague-Dawley rats showed reduced glutamate reuptake by cortical neurons following Aβ exposure (Keller et al. 1997), while TG mice over-expressing mutant hAPP (London mutation) showed reduced astrocyte-mediated glutamate reuptake (Masliah et al. 2000), accompanied by reduced EAAT1 and 2 expression (Matos et al. 2008). Additionally, marked reductions and mislocalization of glutamine synthase have been reported in neurons and astrocytes in AD cases (Robinson 2001; Butterfield et al. 2006) and 3xTg models (Olabarria et al. 2011) implying that glutamate may accumulate in astrocytes. The downstream consequence of reduced clearance and reprocessing of synaptic glutamate may be increased likelihood of excitotoxic damage (Bezprozvanny and Mattson 2008).

Cytoskeletal proteins

Filamentous (F)-actin, the major constituent of the presynaptic bouton cytoskeleton, organises resting SV pools in conjunction with synapsin and can regulate other steps of the SV cycle such as AZ fusion (Morales et al. 2000), clathrin-mediated endocytosis at certain large synapses (Bourne et al. 2006), and activity-dependent

bulk endocytosis (Clayton and Cousin 2009). Because of its ubiquitous expression in many types of synapses (Cingolani and Goda 2008), and its well-characterized involvement in post-synaptic spine dynamics/plasticity (Hotulainen and Hoogenraad 2010), it is hard to isolate the roles of F-actin that are specific to presynaptic boutons in health and disease.

Although only a small fraction of F-actin in dendritic spines and presynaptic boutons is stable, actin stability is crucial for structural synaptic integrity, and is regulated dynamically by actin-stabilizing signalling pathways (Cingolani and Goda 2008). One such pathway is mediated by the small GTPases Rac and Rap and their downstream effectors such as PAK (Penzes et al. 2003). Recently it has been suggested that PAK expression is downregulated in AD (Zhao et al. 2006) resulting in a host of aberrant downstream cytoskeletal changes (Bamburg and Bloom 2009), possibly including an increase in cofilin phosphorylation (Zhao et al. 2006). Cofilin, one of the major regulators of F-actin dynamics (Kuhn et al. 2000), can truncate and depolymerise F-actin if phosphorylated. Furthermore, excess cofilin can cause the formation of cofilin-actin rods, a common feature of neurodegenerative disease inclusions, particularly in AD (e.g. Hirano bodies; Minamide et al. 2000). Such decreases in F-actin stability may disrupt its many roles in presynaptic function, and trigger dendritic spine retraction and collapse post-synaptically.

Trans-synaptic interaction

Neurons also interact via cell-surface adhesion molecules (CAMs) on the pre- and post-synaptic membranes (Figure 1.1), which keep the AZ and PSD tightly aligned and mediate synapse formation, specificity, maturation, function and plasticity (Dalva et al. 2007; McMahon and Diaz 2011). The most extensively studied

presynaptic CAMs are the neurexin family, coded by three separately-regulated genes that give rise to over 1000 isoforms (Baudouin and Scheiffele 2010). This vast arsenal of splice variants is regionally regulated in the brain and is modulated by activity, providing a plausible mechanism for regulating specificity of synaptic connections (Sudhof 2008). Neurexins form trans-synaptic linkages with a variety of post-synaptic binding partners, the most common of which are the neuroligins (Sudhof 2008; Baudouin and Scheiffele 2010).

Recently both neurexins and neuroligins have been implicated in AD pathogenesis. Firstly, expression of CNTNAP2, a neurexin implicated in autism, is reduced in AD brains (van Abel et al. 2012). Moreover, two recent studies have reported that neurexin 3β is sequentially processed by the α- and γ-secretases which process APP, and fAD-linked mutations in γ-secretase cause abnormal intracellular accumulations of neurexin 3β (Bot et al. 2011; Saura et al. 2011). In turn, this may perturb interaction with neuroligins: a recent study found that Aβ binds to neuroligin-1 on excitatory post-synaptic membranes with very high affinity, where it seeds further Aβ oligomerization (Dinamarca et al. 2011). In addition, neuroligin-1 expression in ApoE-ε4 transgenic mice is reduced (Zhong et al. 2008). Overall, these studies suggest that neurexin-neuroligin interaction may be sabotaged by Aβ in AD, and warrant further research on the role of CAMs in AD pathogenesis.

Presynaptic facilitation: a likely physiological role for Aβ?

APP is expressed by all neurons, and its activity-dependent metabolism at presynaptic terminals strongly suggests that it may have a physiological role in synaptic transmission. This APP metabolism secretes Aβ, and a range of recent studies have conclusively shown that physiological concentrations of Aβ can directly

modulate synaptic transmission and plasticity in a dose-dependent, plausibly regulatory role. Low doses of Aβ (pM range) increase the number of vesicles available at the presynaptic AZ (Puzzo et al. 2008; Parodi et al. 2010) and enhance glutamate (Cuevas et al. 2011) but not GABA release (Abramov et al. 2009). This potentiating effect was specific to neurons with low basal activity (Abramov et al. 2009) and is likely mediated by activation of presynaptic α7-nAChRs which have previously been shown to directly bind to Aβ (Dineley et al. 2002). Indeed, blockage of AChRs or genetic ablation of α7-nAChRs decreased Aβ levels and prevented Aβ-induced synaptic facilitation (Puzzo et al. 2011). Furthermore, there are *in vivo* deficits in synaptic plasticity and signalling due to abnormally low Aβ levels in mice deficient for APP (Seabrook et al. 1999), PS1 (Saura et al. 2004) or BACE1 (Laird et al. 2005). These results suggest that regulation of neurotransmitter release at the presynaptic level could be an important physiological role of Aβ. However, by the same token, increases in Aβ concentrations above physiological levels (nM range) by altered/increased APP processing in familial AD, or reduced Aβ clearance in sporadic AD, could result in over-stimulation of SV release.

Summary of presynaptic effects of Aβ

The accumulation of Aβ in AD and TG mice can impact presynaptic dynamics at virtually all stages. Regulators of SV exocytosis such as synaptophysin, SNAREs and other associated proteins are decreased in AD, likely resulting in more spontaneous, asynchronous neurotransmitter release. Eventually, this will lead to SV depletion, as previously suggested (Kelly and Ferreira 2007; Parodi et al. 2010). Exacerbating this, reductions in endocytosis mediators such as AP-2, AP-180 and dynamin lead to reduced SV recycling in AD. The maintenance of different SV pools

is also perturbed via excessive SV release and loss of SV reserves due to synapsin loss and F-actin depolymerisation in AD. There are also alterations in endosomal trafficking that could affect not only retrograde transport of neurotrophin receptors and normal SV recycling, but also increase BACE1-mediated amyloidogenic APP processing. Glutamate release is particularly adversely affected due to decreases in VGlut1 on SVs and reduced glutamate reuptake from synapses via EAATs. Increased Aβ levels may also severely impair its proposed physiological function in facilitating presynaptic signalling, and trigger aberrant homeostatic responses. Therefore, multiple aspects of presynaptic function are affected by Aβ, supporting the view that synaptic dysfunction is integral to AD pathogenesis.

1.5.3 Postsynaptic effects of Aβ

In addition to its myriad presynaptic effects, Aβ overload can impair the postsynaptic side as well. Normal postsynaptic function requires the precise localization and timely recruitment of specific receptors, downstream signalling molecules and enzymes to highly-specialized microdomains on the surface of dendritic spines. Elevated Aβ concentrations result in the loss of key structural elements critical for receptor docking, signal transduction and structural integrity. This potentiates dendritic spine collapse, particularly at regions with high Aβ plaque load.

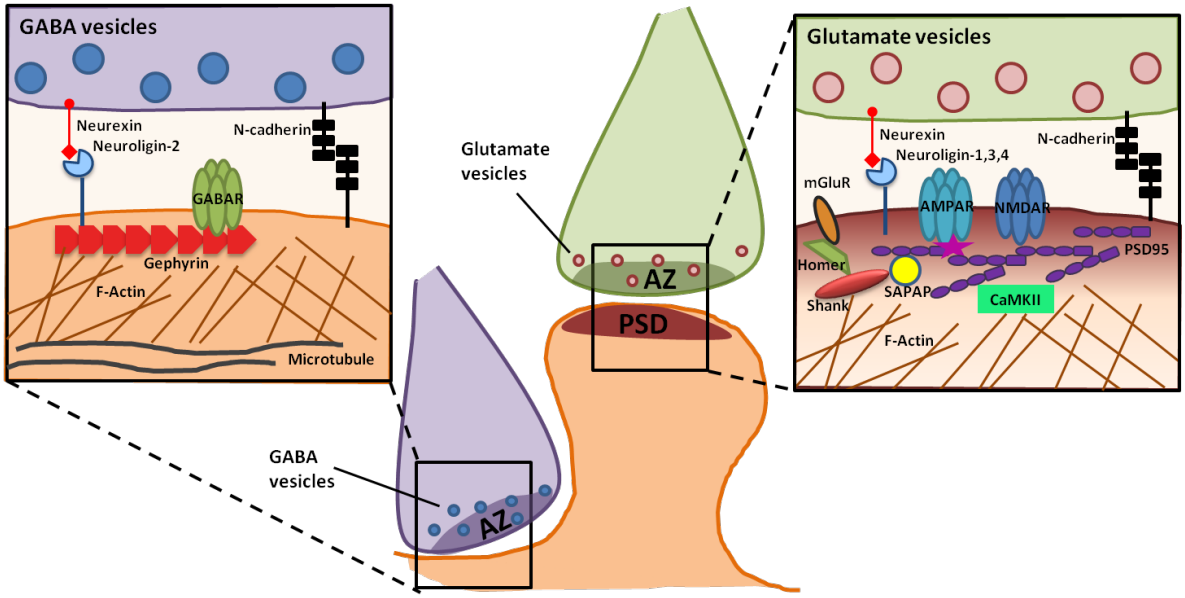
Organization and function of the excitatory postsynaptic densities

There are two types of neurotransmitter receptors: ligand-gated ion channels (ionotropic receptors) and G protein-coupled (metabotropic) receptors. In general, the binding of glutamate or aspartate to amino-3-hydroxy-5-methyl-4-isoazolepropionate (AMPA), N-methyl-D-aspartate (NMDA) or kainate ionotropic receptors enacts excitatory synaptic transmission, and parallel activation of

metabotropic glutamate receptors (mGluRs) elicits intracellular signalling cascades. NMDA receptor (NMDAR) activation permits calcium entry, and plays a pivotal role in mediating postsynaptic plasticity responses. It can induce either long-term potentiation (LTP) or depression (LTD) depending on the magnitude of the intracellular calcium ($[Ca^{2+}]_i$) rise in the dendritic spine following receptor activation (Kullmann and Lamsa, 2007). Large increases in $[Ca^{2+}]_i$ are required for LTP, whereas lower increases due to internalization of synaptic NMDARs, activation of extra-synaptic NMDARs and/or metabotropic glutamate receptors (mGluRs), are necessary for LTD. LTP results in recruitment of AMPARs to the synapse, and growth of dendritic spines, while LTD leads to receptor internalisation, spine shrinkage and synaptic loss (Murakoshi and Yasuda, 2012).

The accumulation of glutamatergic receptors, signalling enzymes and scaffolding proteins at the postsynaptic terminal creates an electron-dense submembranous structure known as the post-synaptic density (PSD) that is directly apposed to the presynaptic AZ across the synaptic cleft (Figure 1.2). In the PSD a dynamic, heterogeneous cluster of receptors and signalling molecules is organised by highly-specialized scaffolding proteins– such as PSD-95, shank and homer– which immobilize receptors to the membrane and offer binding sites for secondary messenger proteins (Sheng and Hoogenraad, 2007).

Figure 1.2 Structural elements of the postsynaptic membrane (adapted from Van Spronsen and Hoogenraad, 2010).



By far the most studied of the scaffolding proteins is PSD-95. Originally described as a binding partner for NR2 subunit-containing NMDARs (Kornau et al. 1995), it is now known to interact with a host of other scaffolding proteins, signalling enzymes and cytoskeletal components (Kim and Sheng, 2004), making it the central organizer of the glutamatergic PSD. It is abundant throughout the PSD, usually close (~12nm) to the postsynaptic membrane (Valtschnoff and Weinberg, 2001; Cheng et al. 2006; DeGiorgis et al. 2006)– ideally placed to interact with cytoplasmic domains of receptors and couple signals to downstream signalling pathways. It plays a critical role in synaptic plasticity by clustering NMDARs at the postsynaptic membrane and regulating their internalization (Roche et al. 2001). More recently, it has also been implicated in AMPA receptor localization and mobility during LTP, helping exchange desensitized AMPARs with naïve ones from the extra-synaptic membrane through its interaction with the adaptor protein, stargazin (Bats et al. 2007; Heine et al. 2008). AMPAR membrane trafficking is further mediated by calcium/calmodulin-

dependent protein kinase 2 (CaMKII), which is localized to the PSD via binding to PSD-95.

In addition to its well-documented receptor-anchoring role, PSD-95 has also been shown to associate with other scaffolding proteins such as Shank that mediate dendritic spine dynamics (Figure 1.2; Sala et al. 2001). PSD-95 levels can directly affect presynaptic release probability via retrograde neuroligin-neurexin signalling, determining synaptic strength and formation (Dean et al. 2003). Postsynaptically, PSD-95 also interacts with kalirin-7, a regulator of F-actin assembly (Xie et al. 2007) effectively linking activity-dependent glutamate receptor plasticity to dendritic spine morphogenesis and dynamics.

Also integral to the PSD is the cell adhesion molecule N-cadherin, which helps stabilize the connection between pre- and postsynaptic membranes (Figure 1.2), anchors PSD-95 and physically limits receptors to the centre of the PSD (Saglietti et al. 2007). During LTP, NMDAR mediated Ca^{2+} influx causes N-cadherin monomers to dimerize thus exerting a tighter adhesion by binding more of their trans-synaptic counterparts (Okamura et al. 2004). This is thought to promote spine growth and stability. Apart from cell adhesion, N-cadherins also regulate synaptic plasticity and signalling pathways through their intracellular interaction with catenins (Arikkarth and Reichardt, 2008).

Elevated Aβ impairs excitatory synaptic transmission

As NMDAR activity is critical for LTP, changes in receptor levels or makeup are likely to affect plasticity, and there is evidence for such changes in AD. Protein and mRNA levels of NMDAR subunits NR1 (Hynd et al. 2004a), NR2A and NR2B (Hynd et al. 2004b) are significantly reduced in AD autopsy tissue. Similarly, AMPA

subunits GluR1 (Wakabayashi et al. 1999), GluR2 (Aronica et al. 1998) and GluR2/3 (Ikonomovic et al. 1997) are significantly decreased. Pathologically elevated Aβ levels may also indirectly block NMDARs to shift signalling cascades towards LTD and synaptic loss (Kamenetz et al. 2003; Hsieh et al. 2006; Shankar et al. 2008). This is consistent with reports that Aβ impairs LTP (Walsh et al. 2002; Cleary 2005) and enhances LTD (Hsieh et al. 2006; Li et al. 2009) *in vivo*. However, the mechanisms by which Aβ induces LTD remain unclear: receptor internalization and/or desensitization followed by spine loss have been implicated.

Accumulation of Aβ at synapses both *in vivo* and *in vitro* has been shown to decrease the number of functional NMDARs (Snyder et al. 2005; Lacor et al. 2007) and AMPARs (Almeida et al. 2005; Hsieh et al. 2006) via receptor endocytosis. Specifically, the NR1 and NR2B subunits of NMDARs are thought to decrease due to Aβ suppression of critical CREB and STEP phosphorylation (Snyder et al. 2005). For AMPA receptors, GluR1 (Almeida et al. 2005) and GluR2 (Hsieh et al. 2006) subunits, are rapidly depleted by Aβ-potentiated phosphorylation of Serine880 which induces receptor endocytosis. Interestingly, the effect on AMPARs was preceded by an early decrease in PSD-95, which has been shown to strongly colocalize with Aβ at synapses (Lacor et al. 2004), and hence could partly explain the loss of both receptors following high-concentration Aβ application (see below). Elevated Aβ levels have also been shown to induce caspase-3 mediated AMPAR endocytosis (D'Amelio et al. 2010).

Aβ may also induce LTD indirectly, by overstimulation and desensitization of NMDARs (Shankar et al. 2008) due to chronic glutamate overload. Pathologically elevated Aβ blocks synaptic glutamate reuptake (Li et al. 2009), decreases cultured astrocyte glutamate transporter-1 (GLT-1) levels *in vitro* (Matos et al. 2008); and

may drive observed reductions in high-affinity glutamate transporters in aged transgenic animals (Mookherjee et al. 2011; Schallier et al. 2011). *In vivo* microdialysis has also shown that CSF glutamate levels become elevated following Aβ treatment (O'Shea et al. 2008). Over-accumulation of extracellular glutamate would initially result in activation of synaptic NMDARs, followed by desensitization, and ultimately, synaptic depression.

A further effect of increased glutamate would be spillover and activation of extrasynaptic NMDARs (Hardingham and Bading, 2010) and/or metabotropic glutamate receptors (mGluRs; Li et al. 2009), both of which play a key role in LTD induction. In fact, Aβ has been shown to bind to extrasynaptic NR2B NMDARs (Li et al. 2011), as well as to induce mGluR5 clustering and abnormal activation (Renner et al. 2010), leading to LTD induction.

Aβ–mediated changes at excitatory postsynaptic terminals – scaffolding proteins

At the level of synaptic organisation, reductions in PSD-95 immunoreactivity and protein levels have been well documented in human AD tissue (Gylys et al. 2004; Proctor et al. 2010; Koffie et al. 2012), and transgenic mouse models (Almeida et al. 2005; Koffie et al. 2009; Shao et al. 2011). Moreover, reductions in synaptic PSD-95 are associated with increased aberrant production in the soma, suggesting a transport failure (Shao et al. 2011). Losses in PSD-95 occur very early in AD progression, even for MCI (Sultana et al. 2010), and are most pronounced in high Aβ load regions such as the temporal cortex (Proctor et al. 2010). One potential mechanism is through NMDAR subunit-dependent signalling: Liu and colleagues (2010) have shown that Aβ can directly reduce PSD-95 expression in a time- and dose-dependent manner by suppressing NR2A function in synaptic receptors and activating NR2B on extra-

synaptic receptors. These receptors are coupled to distinct signalling pathways linked with neurodegenerative diseases: neuroprotection and pro-survival for NR2A, and apoptosis and excitotoxicity for NR2B (Hardingham and Bading 2010). In the case of AD, NR2B activation results in downstream induction of caspase-3 activity (Liu et al. 2010), inhibition of LTP (Li et al. 2011) and elevated Aβ production *in vitro* (Borjdi et al. 2010).

Disruption of PSDs by Aβ is exacerbated by decreases in other critical elements such as Shank and Homer in AD tissue (Pham et al. 2010). Gong and colleagues (2009) reported altered expression levels of different Shank isoforms and aberrant ubiquitination as one possible mechanism by which Aβ disrupts their postsynaptic function. Application of Aβ *in vitro* results in very rapid loss of Homer and Shank assemblies in a PSD-95 independent-manner, suggesting multiple pathways may be activated to mediate PSD disruption. GKAP, a critical adaptor for PSD-95 and Shank binding (thus coupling ionotropic and metabotropic receptors), is also rapidly lost from synapses following Aβ application *in vitro* (Roselli et al. 2011).

Aβ–mediated changes at excitatory postsynaptic terminals – plasticity regulators

Other PSD elements are also affected in AD. Karilin-7, which regulates spine density and size by coupling ephrin-B receptor (EphB) signalling with the actin cytoskeleton (Penzes et al. 2001, 2003; Penzes and Jones, 2008), interacts strongly with PSD-95. AD is associated with a downregulation of karilin-7 mRNA expression (Youn et al. 2007; Murray et al. 2012), and EphB2 receptors have recently been shown to undergo early Aβ-mediated degradation *in vitro* (Cisse et al. 2011).

AD also affects CaMKII. This key plasticity regulator phosphorylates numerous targets following NMDAR-mediated calcium entry and activation, ultimately resulting in AMPAR synaptic targeting and LTP induction. There is now considerable evidence that its activity may be severely compromised by Aβ. Reductions of the phosphorylated active form of CaMKII have been reported in both human AD cases (Amada et al. 2005) and transgenic mice (Gu et al. 2009), and redistribution from dendrites to perikarya may constitute an early pathological alteration in MCI patients (Reese et al. 2011). *In vitro* experiments have confirmed that acute Aβ treatment decreases LTP by preventing CaMKII-mediated AMPA receptor phosphorylation and synaptic trafficking (Zhao et al. 2004). However long-term Aβ treatment at near-physiological doses may actually potentiate CaMKII activation, suggesting a regulatory role that may be occluded in AD (Tardito et al. 2007).

Aβ–mediated changes at excitatory postsynaptic terminals – cell adhesion proteins

As stated above, N-cadherins regulate multiple aspects of synaptic plasticity via interaction with cadherins and catenins. Recently N-cadherins have also been shown to promote APP dimerization *in vitro*, thought to enhance Aβ production (Asada-Utsugi et al. 2011) and suggesting a mechanism for the activity dependence of Aβ metabolism (Cirrito al. 2005). Larger synapses with higher basal activity rates would have more N-cadherin and therefore generate more Aβ. However, N-cadherin is processed very similarly to APP – cleavage by α- and γ-secretases releases a C-terminal fragment which translocates to the nucleus and mediates β-catenin signalling (Uemura 2006a, b) – and this may be disrupted in AD. Increased levels of

Aβ in AD could block NMDA-dependent α-secretase activation, preventing its subsequent cleavage of N-cadherin and its nuclear translocation (Uemura et al. 2007). Alternatively, others have suggested that fAD PS1 mutations cause impaired N-cadherin C-terminal fragment 1 cleavage, resulting in build-up and a possible potentiation of Aβ toxicity, perhaps contributing to synaptic dysfunction (Andreyeva et al. 2012).

Modulation of postsynaptic excitatory synaptic transmission: a likely physiological role for Aβ?

The production of Aβ and its secretion into the extracellular space are tightly regulated by neuronal activity *in vitro* (Kamenetz et al. 2003) and *in vivo* (Cirrito et al. 2005). Increased neuronal activity results in increased Aβ secretion at synapses (Kamenetz et al. 2003) and dendrites (Wei et al. 2010), whereas blocking activity has the opposite effect. Such activity-dependent control of Aβ release occurs not only in pathological conditions such as epileptiform activity induced by electrical stimulation (Cirrito et al. 2005), but also during normal physiological processes, such as the sleep-wake cycle (Kang et al. 2009). These findings support a role for APP and Aβ in negative feedback modulation of synapses. In this feedback model, APP metabolism is enhanced by higher synaptic activity, leading to increased Aβ release (nM range) at synapses and diminution of excitatory activity post-synaptically. However, as discussed in the previous section, low levels of activity, and thus lower amounts of Aβ (pM range), result in presynaptic potentiation and facilitation of glutamatergic synapses (Abramov et al. 2009). Therefore, there may be a dose-dependent, bell-shaped relationship between extracellular Aβ levels and synaptic transmission: low levels reduce presynaptic terminal efficacy, intermediate levels

facilitate, and high levels depress postsynaptic transmission. In AD, pathological elevation of Aβ would shift this toward postsynaptic suppression, resulting in LTD.

Organization of the inhibitory postsynaptic sites

Inhibition in the cortex is mainly carried out by γ-aminobutyric acid type A receptors ($GABA_ARs$), composed from eight subunit classes (α, β, γ, δ, ε, π, ρ, θ) in pentameric assemblies with a central pore permeable to chloride ions (Olsen and Sieghart, 2008). Subunit composition can differ vastly between cell types and developmental stages, giving rise to a wide range of receptor properties. Similar to NMDA receptors, $GABA_ARs$ can accumulate both synaptically and extra-synaptically, serving very different roles. The most common synaptic $GABA_ARs$ are formed by α1/2/3-βx-γ2 subunit combinations (Mangan et al. 2005). They respond to brief high concentrations of GABA (mM range), with fast rise time, high amplitude and fast inactivation. Conversely, extrasynaptic receptors containing α4/5/6-β3-δ subunits have higher GABA affinity and longer channel open times (Wei et al. 2003), responding to lower concentrations (μM range) or spillover from the synaptic cleft. They provide the postsynaptic neuron with tonic inhibition that makes it harder to reach the threshold of action potential firing (Farrant and Nusser, 2005).

Unlike excitatory synaptic connections, inhibitory boutons usually synapse directly on the dendritic shaft or spine neck, and lack a prominent postsynaptic thickening. Nevertheless they also have specialized postsynaptic structures organised primarily by gephyrin, a scaffolding protein first described in glycinergic synapses of the spinal cord. Gephyrin is thought to bind directly to $GABA_ARs$ containing α2 subunits (Tretter et al. 2008) to cluster (Yu et al. 2007) and stabilize (Jacob et al. 2005) them. Reducing gephyrin expression compromises the accumulation of

synaptic $GABA_A$Rs containing α2 or γ2 subunits at inhibitory synapses (Essrich et al. 1998; Kneussel et al. 2001; Jacob et al. 2005) and reduces the amplitude of miniature inhibitory postsynaptic currents and whole-cell GABA currents (Levi et al. 2004; Yu et al. 2007). Nevertheless, there is no change in overall protein levels of α2 or γ2 subunits (Kneussel et al. 1999), and receptors containing α1 or α5 subunits do not appear to be affected by gephyrin knockout, suggesting the existence of gephyrin-independent clustering mechanisms (Levi et al. 2004). Given the large number of gephyrin splice variants, and that its synaptic localization and function is tightly regulated by activity (Hanus et al. 2006) and phosphorylation (Zita et al. 2007), gephyrins may play different roles at distinct $GABA_A$R subtypes. For example, different mechanisms regulate gephyrin-mediated $GABA_A$R clustering at perisomatic and axo-axonic inhibitory synapses (Panzanelli et al. 2011).

Like its excitatory counterpart, PSD-95, gephyrin also participates in signalling pathways beyond its main receptor-clustering role. Recently, neuroligin-2 has been shown to directly bind to gephyrin and initiate perisomatic inhibitory synapse formation (Poulopouos et al. 2009), while gephyrin ablation results in loss of neuroligin-2 from synapses (Varley et al. 2011). Interestingly, gephyrin over-expression has little or no effect on the size and number of gephyrin clusters and GABAergic synapses, but causes a significant reduction in glutamatergic PSD size and NMDA receptor density (Yu and De Blas, 2008). Additionally, gephyrin loss from synaptic clusters has been recently shown to down-regulate both VGLUT levels and the frequency of spontaneous and miniature glutamatergic events (Varley et al. 2011). Such heterotypic effects may be mediated by interactions with (and/or competition for) the neuroligin-neurexin cell adhesion molecules which play a critical role in both inhibitory (Kang et al. 2008) and excitatory (McMahon and Diaz

2011) synapse formation. In this regard, gephyrin has been shown to bind neuroligin-1, an excitatory synapse isoform, and thus potentially compete with PSD-95 for its binding (Keith and El-Husseini 2008). This cross-talk may play an important role in establishing excitatory-inhibitory balance.

Aβ–mediated changes at inhibitory postsynaptic terminals

In stark contrast to glutamatergic terminals, GABAergic synapses appear to be largely spared in AD (Bell et al. 2006). This is consistent with reports of unaltered GABA concentrations in autopsy AD tissue (Lowe et al. 1988; Siedl et al. 2001) and in CSF of AD patients (Jimenez-Jimenez et al. 1998). Moderate reductions in radiolabelled-GABA binding sites have been shown in AD brains (Chu et al. 1987; Vogt et al. 1991), while others found no reductions using PET imaging (Meyer et al. 1995). Aβ released at excitatory synapses binds to NMDA receptors and neuroligin-1 to induce reversible local synapse loss, but it does not bind to $GABA_A$ or neuroligin-2 at inhibitory synapses (Lacor et al. 2007; Shankar et al. 2007; Dinamarca et al. 2011). $GABA_A$ receptor clusters tend to be relatively spared in AD (Rissman et al. 2007) although Agarwal and colleagues (2008) have shown reduced gephyrin protein levels in AD post-mortem tissue compared to controls. Paradoxically, in cases with more severe pathology, gephyrin levels were less reduced than in moderate AD, suggesting that they may be upregulated to compensate for excitotoxic damage at advanced stages of the disease. It is however too early to speculate at this stage given the paucity of data on gephyrin levels in AD.

Even if overall inhibitory synaptic density is preserved, GABAergic function strongly depends on receptor subunit composition, which is also affected by AD (Rissman and Mobley 2011; Table 1.3). The pattern of disruption is complex.

Western blotting of AD brains shows a 13.5% reduction in α5 but no change in α1, β1 and β2 subunits with progression of the disease (Rissman et al. 2003). Immunohistochemical studies of the AD hippocampus show unaffected β2/3 subunits even in advanced disease, but occasional reductions in α1 subunits (Mizukami et al. 1997a; Mizukami et al. 1998b). However, in situ hybridization in highly-affected hippocampal regions, showed reduced β3 and entirely unaffected β2 subunit mRNA (Mizukami et al. 1998a). More recent work has also shown that γ1/3 and γ2 subunits were preserved and in some cases, even elevated in AD hippocampus compared to controls (Iwakiri et al. 2009). Interestingly, neurons with higher γ-subunit expression lacked NFT pathology, suggesting a protective role.

Table 3.3 Alterations in hippocampal $GABA_A$ receptor subunits in AD.

Subunit	**Assay**	**Effect**	**Reference**
α1	Protein	Decrease	Mizukami et al. 1998a
β2/3	Protein	No change	Mizukami et al. 1997b
β2	mRNA	No change	Mizukami et al. 1998b
β3	mRNA	Decrease	Mizukami et al. 1998b
α1, α5	mRNA	Decrease	Rissman et al. 2004
α5	Ligand binding	Decrease	Howell et al. 2000
α5	Protein	Decrease	Rissman et al. 2003
α1, β1, β2	Protein	No change	Rissman et al. 2003
γ2, γ1/3	Immunohistochemistry	Increase	Iwakiri et al. 2009

It may be that GABAergic signalling is actively repaired, rather than spared by AD disease processes. Hippocampal neurons can upregulate and sustain a high production of $GABA_A$ subunit β2/3 (Mizukami et al. 1997b) and γ1/3 and γ2 (Iwakiri et al. 2006) subunits up to 30 days after perforant pathway lesion; similar

compensatory mechanisms may sustain $GABA_A$ receptor subunit levels during the AD process. Interestingly, preserving $GABA_A$ receptor function may have protective effects in AD: selective $GABA_AR$ agonists have been shown to protect cultured neurons from Aβ toxicity (Gu et al. 2003; Lin and Jun-Tian 2003; Louzada et al. 2004; Lee et al. 2005a). Conversely, blocking $GABA_AR$ function with antagonists ablates this protective response and predictably exacerbates Aβ toxicity (Marcade et al. 2008; Tampellini et al. 2010). Protective effects are likely mediated by normalizing the Cl^- flux in postsynaptic neurons which may be perturbed by Aβ (Sawada and Ichinose 1996; Lee et al. 2005a). In addition, $GABA_AR$ agonists shift APP processing to the non-amyloidogenic α-secretase-mediated cleavage, potentially reducing local Aβ production (Marcade et al. 2008).

In summary, AD-related loss of cholinergic and glutamatergic fibres may trigger GABAergic compensation, and there are certainly changes in the GABA system: despite overall sparing of $GABA_A$ receptors in AD, changes in subunit composition (e.g. α1, α5 and β3), and altered receptor function (Rissman et al. 2007; Limon et al. 2012) may have significant impact.

Summary of postsynaptic effects of Aβ

The production and secretion of Aβ from presynaptic sites in an activity-dependent manner suggests that it may also have physiological postsynaptic roles. Indeed, Aβ can directly bind to both synaptic and extrasynaptic glutamatergic NR2-subunit containing NMDARs and mediate local plasticity responses. Interestingly it does not seem to bind GABAergic postsynaptic receptors. Pathologically, Aβ impairs glutamatergic signalling by disrupting functional and structural features of the PSD: it induces LTD and receptor internalization, as well as loss of scaffolding proteins such as PSD-95. It should be noted, however, that some of these changes might be

masked/brought on by the very loss of synapses that they herald. Future studies should therefore aim to clarify the extent of synapse loss already present in the tissue/culture dish and how that correlates to the measured change. Although inhibitory synapses may not be directly targeted by Aβ, compensatory changes resulting from its actions on glutamatergic synapses may also result in adaptive/aberrant function, particularly due to altered receptor subunit expression.

1.5.4 Loss of dendritic spines is accentuated near Aβ plaques

Dendritic spines – small membranous protrusions that contain the PSD, an actin cytoskeleton and a variety of organelles (Sheng and Hoogenraad, 2007) – are highly dynamic, changing size and shape over timescales of seconds to several days (Fischer et al. 1998; Holtmaat and Svoboda, 2009). Such dynamic changes are closely linked to changes in synaptic strength – larger spines often correlate with larger PSDs and presynaptic AZs (Bourne and Harris, 2008) – and depend on neuronal activity and glutamate receptor activation. Induction of LTP causes enlargement of spine heads, whereas LTD induction causes spine head shrinkage and/or retraction (Yuste and Bonhoeffer, 2001).

In AD, Aβ-mediated presynaptic terminal dysfunction and aberrant LTD induction at postsynaptic terminals combine to cause dendritic spine loss. Numerous *in vivo* multiphoton real-time imaging studies in transgenic mouse models show a dynamic, reversible decrease in dendritic spine density and size that is particularly accentuated near Aβ plaques (Tsai et al. 2004; Spires et al. 2005; Grutzendler et al. 2007; Knafo et al. 2009). Remaining spines have reduced stability, with an increased rate of elimination and unchanged rate of spine formation in proximity to plaques (Spires-

Jones et al. 2007). In human AD, early dendritic spine loss correlates well with clinical stage and cognitive status (Akram et al. 2008).

Given the importance of the cytoskeleton, particularly F-actin, in regulating spine dynamics and morphology (Fischer et al. 1998), AD effects on cytoskeletal signalling pathways and enzymes are also relevant. Significant reductions in spine actin-binding proteins such as drebrin 1 (Aoki et al. 2007; Counts et al. 2012) and synaptopodin (Reddy et al. 2005; Arnold et al. 2013) have been reported in both human post-mortem cases, as well as in transgenic mice. In addition, increased cofilin activity in AD has also been reported and may lead to increased F-actin depolymerisation (Zhao et al. 2006). One proposed mechanism for this destabilizing effect on actin is increased calcineurin activity (Wu et al. 2010). Compelling in vitro experiments have now shown that Aβ can elevate neuronal calcium levels to cause rapid aberrant calcineurin activation in spines, which in turn affect spine morphology and numbers (Wu et al. 2012). Such alterations will decrease spine stability and contribute to Aβ mediated synaptic toxicity.

1.5.5 Network-wide effects of Aβ

Most of the synaptotoxic effects of Aβ seem to be selective for the glutamatergic synapses where it is secreted and accumulates, but the resultant disruption would drive compensatory changes in the relatively spared inhibitory system. Indeed, several studies have shown altered inhibitory responses in hippocampal circuits following Aβ toxicity. Increased sprouting and enhanced synaptic inhibition of inhibitory axonal terminals have been observed in the molecular layer of the dentate gyrus, while alterations in activity-regulated proteins including calbindin, c-Fos and Arc have been reported in granule cells (Palop et al. 2003, 2005, 2007; Chin et al.

2005). Although these compensatory mechanisms may dampen pathological increases in network activity, they may also interfere with normal synaptic function and plasticity required for learning and memory, therefore also contributing to cognitive impairment.

Aβ's effects on NMDA at individual synapses have larger scale consequences for networks, such as disruption and aberrant activity (Palop and Mucke 2010). This is beautifully exemplified by *in vivo* calcium dye imaging showing higher proportions of both hyperactive and hypoactive neurons in Aβ plaque-rich transgenic mouse cortex (Busche et al. 2008). Physiologically, such network imbalances may underpin increased epileptogenic activity in human AD cases (Larner 2010) and transgenic mice (Palop et al. 2007), exacerbating cognitive decline. Interestingly, Busche and colleagues (2008) proposed that the increase in hyperactive neuron density was due to decreased inhibition rather than increased glutamatergic signalling. This would suggest, contrary to tissue-level data on synaptic proteins, that GABAergic signalling may be locally perturbed in proximity to plaques *in vivo*. Recently, L-type Ca^{2+} channels (Yang et al. 2009) and Nav1.1 subunit-containing voltage gated sodium channels (Verret et al. 2012) on inhibitory interneurons have been identified as possible targets of Aβ-mediated down-regulation in transgenic mice. As both mediate interneuron activity and plasticity, reductions in such channels could indeed lead to disrupted GABAergic signalling, particularly in areas with high Aβ load. This, however, contradicts the established view that the GABAergic system is relatively spared in human AD and warrants further investigation, particularly in human tissue.

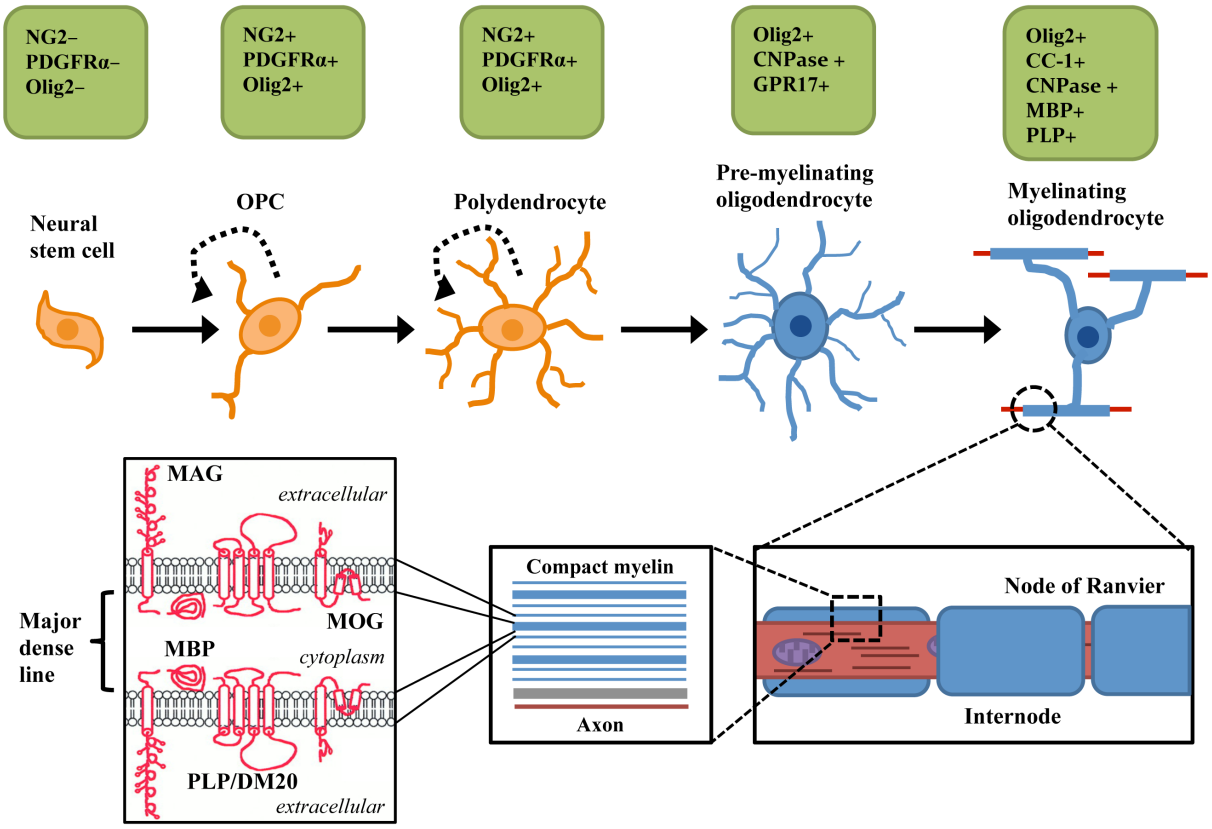
1.6 Myelin pathology in AD

Although glial cells outnumber neurons in the human cortex approximately 4 to 1 (Azevedo et al. 2009), they have traditionally been regarded as secondary players compared to neurons when it comes neurodegenerative processes such as AD. However, it is increasingly apparent that glia not only play a crucial role in the normal functioning and maintenance of CNS neurons, but also a direct and principal part in the pathogenesis of neurodegenerative disorders, as evidenced by new findings in amyotrophic lateral sclerosis and multiple sclerosis. Recent studies have documented roles for both astrocytes (reviewed in: Verkhratsky et al. 2011) and microglia (reviewed in: Ransohoff and Perry 2009) in AD, however, for the scope of this thesis, we shall primarily consider oligodendrocytes, the chief myelinating cells of the CNS.

1.6.1 Basic function and composition of myelin

Since its initial discovery by Rudolf Virchow in 1854, our growing understanding of the structural and functional complexities of the myelin sheath has firmly established its critical role in normal brain function. The myelin sheath is a specialized, insulating extension of the plasma membrane of oligodendrocytes in the CNS (and Schwann cells in the PNS) that wraps around the axis of axons multiple times, giving it a multilamellar appearance (Figure 1.3).

Figure 1.3 Differentiation of oligodendrocytes and organization of compact myelin sheaths.



CC-1, Adenomatous polyposis coli–clone CC1; *CNPase*, 2',3'-Cyclic-nucleotide 3'-phosphodiesterase; *GPR17*, G protein-coupled receptor 17; *MAG*, myelin-associated glycoprotein; *MBP*, Myelin basic protein; *MOG*, Myelin oligodendrocyte glycoprotein; *NG2*, Neuron-glia proteoglycan 2; *Olig2*, Oligodendrocyte lineage transcription factor 2; *PDGFRα*, Platelet-derived growth factor receptor α; *PLP/DM20*, Proteolipid protein 1

Classically, myelination allows for saltatory conductance by clustering Na^+ channels at nodes of Ranvier, which greatly speeds up nerve impulses in a myelinated fibre compared to an unmyelinated fibre of the same calibre (Kaplan et al. 2001). Because it greatly lowers capacitance, it also allows for thinner, more compact fibres that require much less energy to conduct impulses (Bakiri et al. 2009). Furthermore, the physical restriction in the size of active membrane exposed to the outside imposed by myelination, greatly reduces ephaptic cross-talk between adjacent nerve fibres and improves signal specificity.

Although myelination commences during gestation in humans, myelin production peaks post-adolescence– a dynamic process that spans many decades (Benes et al. 1994; Kemper 1994). It is therefore likely that, in addition to intrinsic factors that regulate region-specific myelination in the brain, it can be an adaptive response. For example, learning to play the piano has been linked with increases in fibre tract myelination that is directly commensurate with the time spent on the learning task (Bengtsson et al. 2005). More recently, other tasks such as learning to read (Carreiras et al. 2009) or training to juggle (Scholz et al. 2009), have also been shown to directly increase myelination in specific brain regions. Therefore myelination may be a form of long-term plasticity that is critical for learning and cognition.

Myelin is composed of water (~40%), phospholipids (70-85% of dry weight) and proteins (15-30% of dry weight). The two major proteins of CNS myelin, myelin basic protein (MBP) and myelin proteolipid protein (PLP), account for over 80% of the protein content. The MBP gene gives rise to a large family of isoforms with many post-translational modifications. MBPs are thought to play a crucial role in helping fuse the cytoplasmic interface of myelin lamellae together, forming the major dense line (Figure 1.3; Harauz et al. 2009). MBP knock-out 'Shiverer' mice show decreased amounts of CNS myelination and a progressive disorder characterized by seizures, tremors, and early mortality (Duncan et al. 2011). PLP is the major protein constituent of CNS myelin and is thought to stabilize the multilamellar organization of compact myelin and participate in forming the intraperiod line spacing (Yool et al. 2001). It has one major isoform, DM20, which is also relevant for CNS myelination (McLaughlin et al. 2002). Mutations in the PLP gene result in the unravelling of myelin and axon degeneration such as the 'Jimpy'

mouse, as well in many human conditions such Pelizaeus-Merzbacher disease (Nave et al. 1986).

Myelin-associated glycoprotein (MAG) is also an abundant protein in both CNS and PNS myelin. It is present in the non-compact myelin sheets near the periaxonal membrane of myelinating internodes and is crucial for many axon-glial interactions (Quarles 2007). Its expression is not necessary for formation of compact myelin (Yin et al. 1998). However, MAG-mediated signalling plays a crucial part in establishing axonal calibre via phosphorylation of neurofilaments (Dashiell et al. 2002; Garcia et al. 2003) as MAG knock-out animals have fewer and thinner axons, despite normal myelination (Yin et al. 1998). Moreover, a recent study has also shown that MAG protects axons from injury and prevents axonal degeneration both *in vitro* and *in vivo* (Nguyen et al. 2009). Another abundant protein found specifically in the cytoplasm of non-compact myelin is the enigmatic enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase). Although the reaction it catalyses *in vitro* is well-characterized, its physiological role *in vivo*, and its potential substrates are poorly understood (Kursula 2008). Interestingly, although CNPase knock-out mice seem to produce functional compact myelin, they develop early pronounced axonal swellings and degeneration throughout the CNS (Lappe-Siefke et al. 2003). This suggests that the role of CNPase could be in the trophic support of axons as opposed to myelinogenesis per se. In further support of this, CNPase has been found to interact with the microtubule cytoskeleton and promote process outgrowth in vitro (Lee et al. 2005b).

1.6.2 Oligodendrogenesis and myelination

The process of myelination is very finely tuned by mature oligodendrocytes that undergo successive cycles of demyelination and remyelination in order to optimize myelin thickness in discrete myelin segments (Piaton et al. 2010). For example, thalamocortical fibres originating from different sites of the thalamus can alter their myelin sheath thickness (by removing excess myelin or adding more) so that despite the different distances traversed, signals can arrive contemporaneously (Salami et al. 2003). Such dynamic processes necessitate ongoing communication between axons and oligodendrocytes, as well as oligodendrocyte precursor cells (OPCs). There are multiple signalling pathways within oligodendrocytes as well as external factors such as neuronal activity, neurotrophins and circulating chemokines that can affect OPC migration, differentiation and ultimately initiate myelin production (Nave and Trapp 2008).

During development, OPC migration is guided by multiple regulatory signals. Secreted molecules such as growth factors (e.g. FGF, PDGF; Hu et al. 2012), chemotrophins (e.g. netrins, semaphorins; Xiang et al. 2012) and chemokines (e.g. CXCL1; Patel et al. 2012) all have motogenic effects on OPCs. Likewise, contact-mediated interactions with extracellular matrix proteins (e.g. tenascin C; Czopka et al. 2010), cell-surface molecules (e.g. neuregulins; Ortega et al. 2012) and even physical forces in the neuropil (Bauer and Ffrench-Constant 2009) are also important regulators of OPC migration. Once located at their final destination, some OPCs persist into adulthood while the majority differentiates into myelin-producing oligodendrocytes expressing specific markers such as CC-1, MBP and PLP (Figure 1.3). Adult OPCs express the neuron-glia proteoglycan 2 (NG2) and the platelet-

derived growth factor receptor α (PDGFRα) and are variously called NG2-expressing OPCs (Franklin and Ffrench-Constant 2008), synantocytes (Butt et al. 2005), or polydendrocytes (Nishiyama et al. 2009). Recent evidence from transgenic mice suggests that these adult progenitor cells may not be committed to just the oligodendrocyte lineage but may also give rise to neurons in the hippocampus or piriform cortex (Rivers et al. 2008; Guo et al. 2010), astrocytes (Zhu et al. 2008; Guo et al. 2009), and even Schwann cells (Zawadzka et al. 2010). Conversely, others claim that expression of NG2 restricts OPCs to the oligodendrocyte lineage and they do not produce neurons or astrocytes under normal or traumatic conditions (Kang et al. 2010; Richardson et al. 2011).

Differentiation of OPCs into adult oligodendrocytes is also a tightly regulated process that requires multiple forms of axon-oligodendrocyte signalling (Nave and Trapp 2008; Piaton et al. 2010). Initiation of differentiation involves signalling between the Notch 1 receptor (on oligodendrocyte surface), its ligand Jagged 1 (on the axonal surface) and γ-secretase cleavage of Notch 1 (Watkins et al. 2008; Zhang et al. 2009). In turn, the initiation of myelination is critically controlled by the activity of target neurons (Demerens et al. 1996). For example, unmyelinated neurons synapse directly onto OPCs, but not mature oligodendrocytes (Kukley et al. 2010). OPCs receive both glutamatergic (Kukley et al. 2007; Zizkin et al. 2007) and GABAergic (Wang et al. 2003) inputs and possess ionotropic (De Biase et al. 2010) and metabotropic (Luyt et al. 2003) glutamate receptors, as well as $GABA_A$ (Lin and Bergles 2004) and $GABA_B$ (Luyt et al. 2007) receptors. Furthermore, some OPCs even have the capacity to fire action potentials, likely reflecting that subtypes of OPCs have additional functional roles besides myelination (Karadottir et al. 2008). Aside from direct synaptic influence, action potential firing by neurons results in the

release of ATP (Wake et al. 2011) and adenosine (Stevens et al. 2002), which in turn promotes the release of leukaemia inhibitory factor from astrocytes (Ishibashi et al. 2006), which stimulates myelination while inhibiting OPC proliferation (Fields and Burnstock 2006). OPCs are also electrically coupled to other oligodendrocytes (Maglione et al. 2010) and astrocytes (Tress et al. 2012) in connexin gap junction networks. Such activity-dependent initiation of OPC differentiation and myelination is not only important during development but has also been shown to play a critical part in adult CNS remyelination (Etxeberria et al. 2010).

1.6.3 Consequences of myelination

Besides optimising axonal propagation, myelination confers many other advantages. Fast axonal transport (Edgar et al. 2004) and microtubule stability (Kirkpatrick et al. 2002) are critically dependent on proper myelination. Conditions that disrupt myelin sheath integrity, such as mutations in the PLP gene, result in alterations in microtubule and transport dynamics, often leading to axonal swelling and degeneration, even in the absence of overt demyelination (Garben et al. 2002; Lappe-Siefke et al. 2003). Moreover, myelinating oligodendrocytes provide trophic support for axons by releasing various neurotrophins such as glial cell line-derived neurotrophic factor (GDNF, Wilkins et al. 2003), brain-derived neurotrophic factor (BDNF, Dougherty et al. 2000) and insulin-like growth factor-1 (IGF-1, Du and Dreyfus 2002). Oligodendrocytes also provide metabolic support to axons. For instance, it is well documented that rapidly-conducting white matter axons consume a large fraction of the brain's energy supply and are particularly susceptible to hypoxia and glucose deprivation (Fern et al. 1998). Interestingly, demyelinated axons also have increased numbers of active mitochondria, suggesting higher energy consumption (Andrews et al. 2006). Therefore, it is plausible that oligodendrocytes

alleviate some of this energy demand by actively reducing the amount of energy required for conductance, as well as directly providing ensheathed axons with metabolic support. In this regard, oligodendrocytes have been shown to have enriched expression of monocarboxylate transporter 1, the most abundant lactate transporter in the CNS, likely providing axons with lactate which can be converted to energy (Fünfschilling et al. 2012; Lee et al. 2012). Reductions in this transporter, either by experimental ablation or in neurodegenerative conditions such as amyotrophic lateral sclerosis, results in axonal degeneration and neuron loss. Furthermore, genetic deletion of peroxisome function in oligodendrocytes resulted in late-onset axonal loss without affecting glial survival (Kassman et al. 2007). These findings highlight the importance of normal oligodendrocyte function in supporting axonal metabolism, in addition to the production and maintenance of myelin sheaths.

1.6.4 Demyelination: an early feature of AD?

As myelin is very rich in both lipids and water content, it is particularly amenable to magnetic resonance imaging studies, such as diffusion tensor imaging (DTI-MRI) (O'Dwyer et al. 2011). Diffusion weighted MRI measures the incoherent motion of water molecules for every imaged voxel and provides complementary information to conventional MRI on tissue microstructure. The relatively coherent organization of axons in fibre bundles in the white matter results in a specific pattern of diffusion anisotropy, with greater diffusivity occurring along the axonal direction. Several metrics that characterize different aspects of diffusion tensors (and therefore structural properties of the white matter) have predominantly been analyzed in AD: fractional anisotropy (FA) and mean diffusivity (MD), describing the shape and size of the diffusion tensor respectively, and axial diffusivity (AxD) and radial diffusivity (RD), describing diffusion parallel to the axonal fibres and perpendicular to this main

direction respectively (Pierpaoli et al. 2001; Beaulieu 2002). The latter two are particularly well-suited to describing pathology in AD as decreased AxD is indicative of axonal degeneration (Pierpaoli et al. 2001), while increases in RD are usually associated with breakdown in myelin, which normally acts as a diffusion barrier to water molecules (Song et al. 2002, 2003).

DTI has been used extensively to study white matter pathology in AD. Pathological changes occur very early and mimic the spread of classical AD hallmarks such as NFTs– and to a lesser extent, Aβ plaques– and correlate well with cognitive symptoms (Huang and Auchus 2007; Kavcic et al. 2008). Specifically, medial temporal lobe fibres connecting to and from the hippocampus are affected very early (Salat et al. 2010; Gold et al. 2012). Moreover, tract abnormalities progress from limbic/commissural to association and projection tract groups and include increases in MD and RD, while FA and AxR are uniformly decreased (Huang et al. 2012). Similar patterns of white matter tract abnormalities and atrophy have also been recorded in multiple AD mouse models (Song et al. 2004; Sun et al. 2005; Zerbi et al. 2012). Histological examinations in transgenic mice suggest that myelin loss, decrease in axonal density, and axonal disconnection contribute to the diffusion changes in white matter (Song et al. 2004; Chen et al. 2011b). Likewise, post-mortem analyses in human AD tissue with myelin-staining dyes such as Luxol fast blue, have revealed considerable loss of myelin in frontal, parietal and temporal lobes, but not in the occipital lobe (Sjobeck et al. 2005; Ihara et al. 2010).

It is well established that white matter integrity progressively deteriorates with normal aging (Lindner et al. 2009; Stricker et al. 2009), but evidence from gross brain imaging studies suggests that this natural tendency could be exacerbated and

accelerated in AD (de la Monte 1989; Bartzokis 2004). Furthermore, although white matter lesions are present in both AD and non-demented aging, it has been proposed that, in AD, such lesions may influence the severity of cognitive impairment, especially in the earlier stages of the disease (Burns et al. 2005). The aetiology of subcortical demyelination remains controversial: diffuse white matter lesions have been reported even in the early stages of AD independent of any signs of infarction, white matter amyloid angiopathy, or Wallerian degeneration (Brun and Englund 1986; de la Monte 1989; Bartzokis 2004); whereas strong evidence links age-related ischaemic insults and overall deterioration of cerebrovascular function to white matter damage (Tang et al. 1997, Brown et al. 2000; Fancy et al. 2010). It is important to note however, that there is no direct correlation between the severity of white matter lesions with the overall degree of cortical AD progression (Roher et al. 2002).

Although many CNS axons are myelinated, different neocortical regions myelinate heterochronologically (Yakovlev and Lecours 1967), and late-myelinating regions such as prefrontal and other association areas (temporal and parietal lobes) myelinate very thin axons (Hildebrand et al. 1993) – which are proposed to be those most susceptible to neurodegeneration in AD (Braak et al. 2000; Fornari et al. 2010). Furthermore, oligodendrocyte precursors from later-myelinating regions have reduced myelin turnover and thus a diminished capacity for myelin repair than earlier-myelinating oligodendrocytes (Power et al. 2002). Gross imaging studies report considerable white matter loss associated with AD, particularly in later-myelinating regions such as parts of the temporal and frontal lobes, whereas early-myelinating regions such as the somatosensory and motor cortices are often relatively spared even in very advanced AD cases (Bartzokis 2004; Stricker et al.

2009). This 'retrogenic' pattern of myelin loss parallels the high vulnerability and earlier pathology of cortical association areas compared to sensory regions (Reisberg et al. 1999; Braak et al. 2011). Given myelin's crucial role in mediating the speed and integrity of axonal transmission, it is likely that myelin pathology would significantly contribute to the cognitive decline characteristic of AD.

1.6.5 Mechanisms of demyelination in AD

It is not clear whether plaque-associated demyelination represents a primary event related to Aβ deposition or whether it may follow secondary alterations in axons that lead to dystrophic neurite formation. Desai et al. (2009) have recently suggested that in a triple transgenic mouse model, levels of myelin-associated proteins in the grey matter may be significantly lower than wildtype levels even before overt tau or Aβ pathology is evident. Aβ is also cytotoxic to oligodendrocytes *in vitro*, inducing mitochondrial dysfunction, DNA fragmentation, and eventual death (Xu et al. 2001; Lee et al. 2004; Roth et al. 2005; Chen et al. 2006), but is not harmful to OPCs (Horiuchi et al. 2012). Furthermore, it has been previously reported that there are numerous biochemical alterations in the white matter in AD, such as decreased myelin basic protein and myelin proteolipid protein, as well as a significant reduction in white matter cholesterol levels (Svennerholm and Gottfries 1994; Roher et al. 2002). All of these alterations combined, could severely impair oligodendrocyte function and result in loss of myelin.

Oligodendrocytes could also be acutely vulnerable to certain types of toxicity that are present in AD. During the peak of myelination, it has been estimated that a single oligodendrocyte could myelinate up to 50 internodes (fewer for larger axons requiring thicker myelin), generating up to 150,000μm^3 of myelin (Ludwin 1997)

during its lifespan. This requires the manufacture of up to three times its initial weight in membrane per day, the total eventually reaching as high as 100× the mass of its cell body (Bradl and Lassman 2009). This prodigious effort requires that oligodendrocytes have very high metabolic rates, consuming large amounts of oxygen and ATP (McTigue and Tripathi 2008). The production of ATP in mitochondria inevitably results in the formation of hydrogen peroxide, while a high metabolism creates reactive oxygen species, both of which must be promptly metabolized. Secondly, many of the lipid-synthesizing enzymes involved in myelination require iron as a co-factor, as iron deficiency results in dysmyelination (Todorich et al. 2009). In fact, oligodendrocytes have the highest intracellular stores of iron in the brain, and nearly 70% of brain iron is associated with myelin (Thorburne and Juurlink 1996). Under certain conditions including AD (Smith 2010), elevated intracellular iron levels may lead to free radical formation and lipid peroxidation (Juurlink 1997). Oligodendrocytes are further disadvantaged by their low expression of the anti-oxidative enzyme glutathione (Juurlink 1997), rendering them susceptible to oxidative damage. The large amount of protein synthesis required for myelin assembly also stresses the endoplasmic reticulum of oligodendrocytes, where most post-translational modifications take place. Even small variations in the levels of a single protein (e.g. PLP) may disorganize the whole system and result in the retention, misfolding and accumulation of many other proteins in oligodendrocytes (Bauer et al. 2002).

Oligodendrocytes also express multiple molecules that render them vulnerable to excitotoxic cell death (Fu et al. 2009; Matute 2011): they carry AMPA (McDonald et al. 1998; Ruiz et al. 2010), kainate (Sanchez-Gomez and Matute 1999; Alberdi et al. 2006), and NMDA (Salter and Fern 2005; Micu et al. 2006) receptors which make

them susceptible to glutamate excitotoxicity, as well as the ATP receptor P2X7 which predisposes them to the toxic action of sustained extracellular ATP levels (Domercq et al. 2010). Therefore, there are multiple sources of vulnerability that could render oligodendrocyte particularly susceptible to the toxic effects of Aβ in AD. Aside from the pronounced early alterations in specific white matter tracts, little is known about the involvement of grey matter demyelination and oligodendrocyte loss in AD. Given the evident toxic effects of Aβ on oligodendrocytes *in vitro*, it would be interesting to ascertain if they are also affected *in vivo*.

1.7 Project aims

While the pathological hallmarks of AD and cellular pathways involved in the production, processing and functions of Aβ and tau have been extensively investigated, the degree to which inhibitory and excitatory neurons are affected in AD remains unclear. Likewise, although overall synapse loss is strongly correlated with loss of cognitive function in AD, the susceptibility of inhibitory and excitatory presynaptic terminals to Aβ toxicity in plaque-rich neuropil remains controversial. As inhibition plays a key role in normal conginitve function, deficits in inhibition would likely result in an exacerbation of AD symptoms. It is therefore imperative to investigate the degree of vulnerability of the inhibitory system to Aβ toxicity in AD. Apart form principal and inhibitory cortical neurons, oligodendrocytes, the myelinating cells of the CNS, and astrocytes, which support many neuronal functions, have also been implicated in AD. Loss of white matter myelin has previously been reported in AD tissue, however, very little is known about grey matter demyelination. Myelination is critical for normal axonal development as well as playing an important role in plasticity responses during adult learning. Moreover, oligodendrocytes not only produce myelin, but are also involved in numerous types of neuron-glia signalling and the metabolic support of axons. Loss of oligodendrocytes or myelin structural proteins due to Aβ toxicity could result in demyelination which would severly impact cortical processing and further accelerate cognitive decline in AD. It is therefore the objective of this thesis to address these important issues with the following specific aims.

Aim 1. To determine if different neuron subpopulations are selectively vulnerable in human AD cases and transgenic mouse models of AD

To date, the literature regarding the susceptibility of interneurons to AD cytoskeletal pathology remains inconclusive with early reports suggesting that inhibitory neurons are relatively spared, while more recent data, primarily from transgenic mouse AD models, imply that they are selectively vulnerable early in disease progression. Given the importance of proper interneuron function for providing inhibition in the cortex, it is imperative to investigate the degree to which these neurons are affected by cytoskeletal pathology such as DN formation in AD.

Aim 2. To determine if excitatory and inhibitory presynaptic boutons are differentially affected in proximity to Aβ plaques in human AD cases and in a transgenic mouse model of AD

The balance of inhibition to excitation in the brain is critical for normal cognitive function. There are numerous reports of decreases in glutamatergic presynaptic terminals, decreased glutamate concentrations in the cortex and impaired glutamatergic signalling both *in vitro* and *in vivo* in AD. However, relatively less is known about the degree to which the inhibitory system is involved in AD. Previous reports have suggested that like interneurons, inhibitory synapses are relatively spared in AD. However, more recent reports of epileptic activity and widespread network dysfunction in both AD cases and transgenic mouse models imply that the inhibitory system may be perturbed as well, particularly in the vicinity of Aβ plaques.

Aim 3. To determine if glutamate decarboxylase activity is affected in young and old transgenic AD mice

Apart from bouton density, another key determinant of GABAergic signalling strength is the amount and activity of glutamate decarboxylase (GAD), the chief GABA synthesizing enzyme in the mammalian brain. To date, there have been very few reports of the activity of GAD in transgenic mouse tissue, necessitating a more thorough analysis. Previous reports in human AD tissue have reported limited decreases in GAD activity in subcortical structures, however no changes were reported in the cortex. As GAD catalyzes the rate-limiting step in GABA production, even small changes in its activity could result in large-scale deficits in GABAergic signalling.

Aim 4. To assess if myelin and oligodendrocyte lineage cells are affected in human preclinical, sporadic and familial AD cases, as well as in transgenic AD mice.

Although white matter demyelination and numerous white matter tract myelin abnormalities have previously been reported in AD, very few studies have focused on grey matter myelin integrity in AD. Despite there being significantly fewer myelinated fibres in the cortex, cortical demyelination could significantly contribute to cognitive decline in AD as myelin is crucial for optimizing axonal transport and signal conduction. Indeed, previous reports have shown that regions that myelinate later in development are also more susceptible to AD pathology, suggesting that degree of myelination could predispose certain subpopulations of cortical neurons to AD pathology. Moreover, numerous studies have shown that Aβ is toxic to oligodendrocytes, the chief myelinating cells in the CNS, *in vitro*. Therefore, the

degree to which oligodendrocytes and myelin are affected in AD and transgenic mouse cortical tissue needs to be determined.

2. Materials and Methods

2.1 Human brain tissue source and processing

Human brain tissue was obtained from multiple sources: National Tissue Resource Centre, University of Melbourne (Australia), Sun Health Research Institute (Arizona, USA) and the Department of Pathology, University of Sydney (Australia), as previously described (Dickson et al. 1999; Woodhouse et al. 2009b). Presenilin-1 (PS-1) familial human AD cases were obtained from the Prince of Wales Medical Research Institute (Sydney, Australia) as previously described (Woodhouse et al. 2009a). Permission for brain autopsy and use for research were obtained by the original tissue sources, and the University of Tasmania Human Research Ethics Committee approved all research. Blocks of inferior temporal cortex, a major site of Aβ deposition (Braak et al. 2011), were immersion-fixed with either 10% formalin or 4% paraformaldehyde. Brain tissue was cryoprotected in 18%, then 30% sucrose solution, embedded in Shandon cryomatrix tissue compound (Thermo Scientific, Runcorn, UK) and 40μm thick slices of the inferior temporal gyrus (ITG) were sectioned on a cryostat.

Throughout this thesis, a total of five familial AD cases (PS-1 mutations), eight sporadic AD cases meeting CERAD diagnostic criteria (Braak stage IV-V; Braak & Braak, 1991), eight preclinical AD cases, and five age-matched control cases lacking Aβ plaques and neurofibrillary pathology were used (Table 2.1). With respect to aims 1 and 2, only sporadic AD cases 1-6 and preclinical AD cases 1-6 were utilized. Also used were a subset of non-demented cases with pathological aging including widespread neocortical Aβ plaques, but no 'classical' neurofibrillary pathology or overt nerve cell degeneration (Price and Morris 1999; Vickers et al. 2000). Such

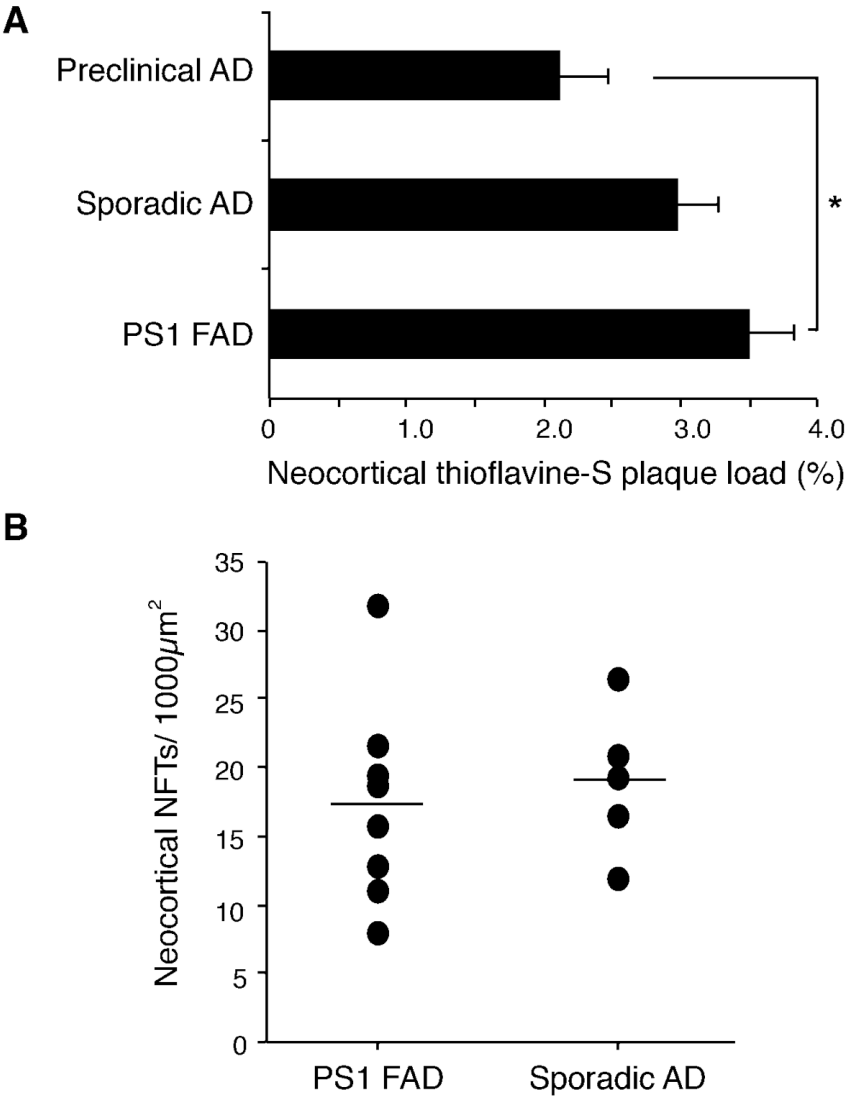
pathologically aged cases correspond to Braak stage III pathology, and may represent a preclinical stage of AD. These cases did not conform to the CERAD criteria for the diagnosis of clinical AD, but their brains exhibit Braak stage III pathology including widespread non-neuritic (based on thioflavin S or PHF-tau-labelling) Aβ plaques in the neocortex and neurofibrillary pathology in the entorhinal formation and hippocampus (Vickers et al. 1996; Price and Morris 1999; Morris et al. 2001).

Table 2.1 Human brain cases utilized for immunohistochemistry and analysis.

Type	Age	Gender	Postmortem interval (h)	Cortical Region	Cause of Death	Source*	Fixation
PS1 FAD	44	F	20.0	TC	Pneumonia	NRA	10% formalin
PS1 FAD	46	F	5.0	TC	Pneumonia	NRA	10% formalin
PS1 FAD	48	M	8.0	TC	Cardiopulmonary arrest	NRA	10% formalin
PS1 FAD	49	M	20.0	TC	Cardiopulmonary arrest	NRA	10% formalin
PS1 FAD	61	F	7.0	TC	Pulmonary embolus	NRA	10% formalin
Sporadic AD	88	M	7.0	ITG	AD	SHRI	4% PFA
Sporadic AD	92	F	2.25	ITG	Pneumonia	SHRI	4% PFA
Sporadic AD	74	F	2.0	ITG	Pneumonia	SHRI	4% PFA
Sporadic AD	74	M	2.75	ITG	Respiratory arrest	SHRI	4% PFA
Sporadic AD	83	M	2.75	ITG	AD	SHRI	4% PFA
Sporadic AD	66	M	2.75	ITG	AD	SHRI	4% PFA
Sporadic AD	84	F	3.0	ITG	AD	SHRI	4% PFA
Sporadic AD	76	M	3.5	ITG	Cardiac arrest	SHRI	4% PFA
Preclinical AD	90	M	2.25	ITG	Respiratory arrest	SHRI	4% PFA
Preclinical AD	81	F	3.0	ITG	Cardiac arrest	SHRI	4% PFA
Preclinical AD	84	M	3.0	ITG	Cardiopulmonary arrest	SHRI	4% PFA
Preclinical AD	78	M	2.25	ITG	Postoperative	SHRI	4% PFA
Preclinical AD	91	M	3.0	ITG	Cardiac arrest	SHRI	4% PFA
Preclinical AD	82	M	48.0	ITG	Myocardial infarction	NTRC	4% PFA
Preclinical AD	74	M	65.0	ITG	Cardiac arrest	NTRC	4% PFA
Preclinical AD	74	M	31.5	ITG	Cardiac arrest	NTRC	4% PFA
Control	58	M	27.0	TG	Cardiac arrest	U Syd	4% PFA
Control	51	M	23.5	TG	Pulmonary embolus	U Syd	4% PFA
Control	47	M	27.5	TG	Pneumonia	U Syd	4% PFA
Control	65	M	16.0	TG	Cardiopulmonary arrest	U Syd	4% PFA
Control	71	M	32.5	TG	Postoperative	U Syd	4% PFA

**NRA*, Neuroscience Research Australia (Sydney, Australia); *SHRI*, Sun Health Research Institute (Arizona, USA); *NTRC*, National Tissue Resource Centre (Melbourne, Australia); *U Syd*, Department of Pathology, University of Sydney (Australia).

Figure 2.1 Gross neuropathological analysis of human tissue used in this thesis[1].



2.2 Histological Stains

2.2.1 Thioflavine S staining

Thioflavine S staining procedures were carried out entirely in lightproof containers. Tissue sections were placed in 0.0125% thioflavine S (Sigma-Aldrich, St Louis, MO)

[1] Data kindly provided by Dr Adele Woodhouse (Woodhouse et al. 2009a). (A) Plaque load was significantly higher in PS-1 AD cases than preclinical AD cases. (B) There was no differene in NFT counts between sporadic and familial AD cases. *$p < 0.05$

dissolved in 60% 0.01M phosphate buffered saline (PBS) and 40% ethanol for three minutes on an orbital shaker at room temperature (RT). Following differentiation in two one minute incubations in 50:50 PBS:ethanol at RT, the tissue sections were washed three times with 0.01M PBS at RT on an orbital shaker. Thioflavine S stains a subset of β-amyloid plaques that are comprised of fibrillar aggregates but not diffuse ones (Dickson and Vickers 2000).

2.2.2 DAPI nucleic acid stain

Tissue sections were incubated with 0.3mM DAPI (Molecular Probes, Invitrogen) in PBS for 10 minutes in the dark at RT, on an orbital shaker. Tissue sections were then washed three times with 0.01M PBS for 10 minutes each, at RT on an orbital shaker.

2.3 Immunohistochemistry

2.3.1 Formic acid epitope exposure

Tissue sections were incubated in 90% formic acid (Sigma-Aldrich, St Louis, MO) for 20 minutes at RT on an orbital shaker. Brain sections were then washed four times with 0.01M PBS for 10 minutes each, at RT on an orbital shaker.

2.3.2 Autofluorescence quenching

To quench autofluorescent signal from lipofuscin granules, human brain sections were stained in 0.25% potassium permanganate for 20 minutes at RT on an orbital shaker and washed twice in 0.01M PBS for two minutes at RT. Sections were then stained in 1.0% potassium metabisulphite and oxalic acid for one to two minutes. Tissue sections were then washed three times with 0.01M PBS for 10 minutes each, at RT on an orbital shaker.

2.3.3 Indirect fluorescent immunohistochemistry

Brain tissue sections were incubated in primary antibody solutions for 16-72 hours at RT or at 4°C (Table 2.2). Optimal concentrations were individually determined for each antibody, and no-primary controls eliminated all immunoreactivity. After three washes with 0.01M PBS for 10 minutes, each at RT on an orbital shaker, primary-labelled sections were incubated with Alexa Fluor goat anti-mouse/rabbit secondary antibodies for two hours at RT, on an orbital shaker in the dark (Table 2.3). All antibodies were diluted in 0.3% triton-X (Sigma-Aldrich, St Louis, MO) in 0.01M PBS to permeabilise the cell membranes. Tissue sections were then washed three times with 0.01M PBS for 10 minutes each at RT on an orbital shaker, and mounted onto microscope slides and coverslipped with PermaFluor aqueous mounting medium (Thermo Fisher Scientific, Rockford, IL).

Table 2.2 Primary antibodies used for immunohistochemistry

Name	Type	Labels	Dilution	Source
anti-CC1	M	Adenomatous polyposis coli; labels mature oligodendrocytes	1:100	Abcam (Cambridge, UK)
anti-calretinin	R	Calcium-bound and unbound conformations of calretinin	1:2000	Millipore (Temecula, CA)
anti-desmin	R	Smooth muscle cells	1:200	Millipore (Temecula, CA)
anti-dMBP	R	Degraded myelin basic protein	1:100	Millipore (Temecula, CA)
anti-GAD65	M	Glutamate decarboxylase 65kDa	1:250	Millipore (Temecula, CA)
anti-GAD67	M	Glutamate decarboxylase 67kDa	1:1000	Millipore (Temecula, CA)
anti-GPRC17	R	Pre-myelinating oligodendrocytes	1:100	Cayman Chemical (Ann Arbor, MI)
anti-MBP	R	Myelin basic protein	1:250	Millipore (Temecula, CA)
anti-NG2	R	Oligodendrocyte precursors	1:100	Millipore (Temecula, CA)
anti-Olig2	R	Oligodendrocyte lineage marker	1:2000	Millipore (Temecula, CA)
Anti-p25α	R	Adult oligodendrocytes	1:50	gift from Professor Poul H Jensen
anti-pan-β-amyloid	R	All β-amyloid peptides	1:1000	Biosource Int (Camarillo, CA)
anti-PDGFRα	R	Oligodendrocyte precursors	1:500	SantaCruz Biotech (Santa Cruz, CA)
SMI312	M	Phosphorylated NFM and NFH	1:2000	Covance (Princeton, NJ)
SMI32	M	Dephosphorylated NFM and NFH	1:2000	Covance (Princeton, NJ)
anti-synaptophysin	R	All presynaptic terminals	1:1000	Millipore (Temecula, CA)
anti-VGAT	M	GABAergic presynaptic vesicles	1:250	Synaptic Systems (Germany)
anti-VGlut1	R	Glutamatergic presynaptic vesicles	1:250	Synaptic Systems (Germany)

Table 2.3 Fluorescent secondary antibodies used for immunohistochemistry.

Excitation (nm)	Reactivity	Species	Dilution	Source
488	mouse IgG	goat	1:500	Molecular Probes (Eugene, OR)
546	mouse IgG	goat	1:500	Molecular Probes (Eugene, OR)
594	mouse IgG	goat	1:500	Molecular Probes (Eugene, OR)
633	mouse IgG	goat	1:500	Molecular Probes (Eugene, OR)
488	rabbit IgG	goat	1:500	Molecular Probes (Eugene, OR)
546	rabbit IgG	goat	1:500	Molecular Probes (Eugene, OR)
594	rabbit IgG	goat	1:500	Molecular Probes (Eugene, OR)
633	rabbit IgG	goat	1:500	Molecular Probes (Eugene, OR)

2.3.4 Antigen retrieval

Brain sections were loaded into tissue cassettes, placed in 0.1M citrate buffer (pH 6.0) and heated on high for 10 minutes in a conventional microwave oven (LG MS-314SCE, 1000 watts). The tissue sections were then heated on high power for a further two and a half minutes, then six minutes on medium power in a pressure cooker (Nordic Ware, Minneapolis). The specimens were cooled to RT in citrate buffer before being transferred back into 0.01M PBS. Tissue sections were then removed from the tissue cassettes and washed three times with 0.01M PBS for 10 minutes each at RT on an orbital shaker.

2.3.5 Indirect immunoperoxidase immunohistochemistry

Brain sections were washed for 15 minutes in 1.0% hydrogen peroxide in methanol, then three times 10 minutes in 0.01M PBS, then incubated in primary antibody solutions for two hours, all at RT on an orbital shaker, and further incubated overnight at 4°C. The sections were then washed three times 10 minutes in 0.01M PBS, and incubated in goat anti-mouse/rabbit immunoglobulin horse-radish peroxidase (1:200, DAKO) for one and a half hours, followed by three 10 minute washes with 0.01M PBS, all at RT on an orbital shaker. All antibodies were diluted in 0.3% Triton-X in 0.01M PBS to permeabilise the cell membranes. The Vectastain

ABC Kit (Vector Laboratories, Burlingame, CA) was used for avidin/biotin amplification according to manufacturer's instructions. The amplified complex was visualised by a two to three minute incubation in Sigma Fast™ 3,3'-diaminobenzidine tablet set (Sigma-Aldrich, St Louis, MO) diluted in Milli-Q® water. The tissue sections were washed three times with 0.01M PBS for 10 minutes each at RT on an orbital shaker, then mounted and left to dry overnight at RT. The mounted slides were then washed in Milli-Q® water for 20 minutes at RT followed by three minute washes in 70%, 90% and 100% ethanol. After 30 minutes in xylene at RT the tissue sections were coverslipped with DPX (Sigma-Aldrich, St Louis, MO).

2.4 Immunoblots

For Western blot analysis of synaptic proteins, fresh or frozen brain tissue samples from APP/PS1 animals and wildtype controls were used. The protein concentration of samples was determined using the Qubit fluorescent protein assay kit (Molecular Probes, Eugene, OR) in triplicate. Samples were then separated by SDS-PAGE (20µg protein/lane; 12% NuPAGE Novex Bis-Tris gels; Molecular Probes, Eugene, OR) using the XCell SureLock™ Mini-Cell gel electrophoresis system (Invitrogen, Eugene, OR). Broad range pre-stained molecular protein standards (PageRuler™ Plus; Thermo Fisher Scientific, Rockford, IL) were also run on each gel to allow protein size determination. Protein gels were transferred to a PVDF membrane (Bio-Rad Laboratories, Berkley, CA), and membranes were blocked for 2 hours in 5% commercial skim milk powder dissolved in Tris-buffered saline with 0.1% Tween-20 (TBST). One gel from each experiment was stained with Coomassie blue (Brilliant Blue R; Sigma-Aldrich, St Louis, MO) to compare replicates for consistency. Blocked membranes were incubated overnight at 4°C in primary antibodies diluted in TBST. Membranes were then washed three times in TBST at RT on an orbital

shaker. Species-appropriate horseradish peroxidise-conjugated secondary antibodies (1:2000; DAKO, Denmark) were applied and visualized with a chemiluminescent peroxidase substrate kit (Millipore, Eugene, OR).

2.5 Microscopy

Immunolabelled specimens were examined using a Leica DM LB2 immunofluorescence microscope or a Leica DMB IRB inverted fluorescence microscope. Images were acquired using a cooled CCD Magnafire (Optronics) digital camera and Magnafire (version 1.0) software. Confocal stacks were acquired with a 63× oil-immersion objective (NA=1.4; Zeiss) using a confocal microscope (Zeiss LSM510) equipped with Zen software and Ar 488, HeNe 543 and HeNe 633 lasers. Scans from each channel were collected individually in multiple-track mode and subsequently merged. Care was taken to use the lowest laser power, and no bleed-through was visible between Alexa 488, Alexa 546 and Alexa 633 channels. Images were acquired using the same acquisition parameters for all samples and were saved as 8-bit TIFF files for analysis with NIH ImageJ software (version 1.45p).

2.6 Data analysis

Statistical analyses for comparisons of group means was conducted by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc tests or unpaired Student's t-tests using GraphPad Prism software (version 5.0b), with p values less than 0.05 (CI 95%) being considered statistically significant. Means were reported ± standard error of the mean (SEM). Graphs were prepared in Microsoft Excel (Office 2011) or Prism (version 5.0b). Images for figures were prepared using Adobe Photoshop (CS5).

All solution recipes are provided in full in Appendix A.

3. Neurites containing the neurofilament-triplet proteins are selectively vulnerable to cytoskeletal pathology in AD and transgenic mouse models

3.1 Introduction

Alzheimer's disease (AD) is commonly associated with a cascade of neuronal cytoskeletal aberrations including the loss of dendritic spines and dystrophic neurite formation, ultimately resulting in neuronal degeneration (Spires et al. 2005; Adalbert et al. 2009; Vickers et al. 2009). These pathological changes develop in a characteristic spatiotemporal progression across the cortex in most AD cases (Braak et al. 2011), and to some extent in AD mouse models (Blanchard et al. 2003), suggesting a differential subregional and cellular susceptibility to AD.

This lab has previously shown that dystrophic neurite formation is accentuated near dense and fibrillar Aβ plaques, and manifests early in AD progression (Dickson et al. 1999; Dickson and Vickers 2001; Woodhouse et al. 2009b). Likewise, loss of dendritic spine density, increase in neurite curvature, sprouting and varicosities in pyramidal neuron dendrites (Le et al. 2001; D'Amore et al. 2003; Spires et al. 2005, 2007; Grutzendler et al. 2007), as well as in cortical axons (Su et al. 1998; Tsai et al. 2004; Adalbert et al. 2009) associated with Aβ plaques have been reported in various mouse models of AD. Since neuron loss in these models is minimal and occurs at later stages, neuronal dysfunction is likely due to neuritic and synaptic degeneration (Adalbert et al. 2009; Woodhouse et al. 2009b). Previous work has also suggested that Aβ plaques can exert a physical effect on surrounding neuronal processes resulting in characteristic dystrophic alterations (Woodhouse et al. 2005). Hence, the effects of plaques on different subpopulations of neurochemically identified neurites

in the plaque core (zone 1), at the plaque periphery (zone 2) and in the surrounding neuropil (zone 3) were examined.

Early stereological studies in AD neocortex supported the view that GABAergic neurons were spared, with only minor decreases in GABA neuron density and size reported (Hof and Morrison 1991; Hof et al. 1991, 1993; Fonseca and Soriano 1995; Sampson et al. 1997; Leuba et al. 1998). However, more recent reports have shown losses in subpopulations of GABA neurons (Koliastos et al. 2006; Baglietto-Vargas et al. 2010; Takahashi et al. 2010). Given the importance of inhibitory input to maintain normal neuronal function and coordinate network activity, it is clearly important to determine the degree of GABAergic neuron susceptibility to AD pathology.

Therefore, a morphological analysis of calretinin- and neurofilament triplet-immunoreactive (CR-ir and NF-ir, respectively) neurons in early and end-stage human AD cases and in Tg2576 and APP/PS1 mice was performed, to compare their susceptibility to neurite loss and dystrophic neurite formation, particularly near fibrillar Aβ plaques.

3.2 Materials and Methods

Human tissue

Human tissue included six sporadic AD cases, six 'preclinical' AD, and five age-matched non-demented cases lacking Aβ plaques or neurofibrillary pathology (see Chapter 2).

Mouse tissue

Twelve-month-old Tg2576 (APP_{Swe}) (Hsiao et al. 1996), APP/PS1 (APP_{Swe}×$PS1_{M146L}$) (Borchelt et al. 1997) and age-matched wildtype (C57BL/6) mice (*n*=5, for each strain) were used immunohistochemical analysis.

Immunohistochemistry

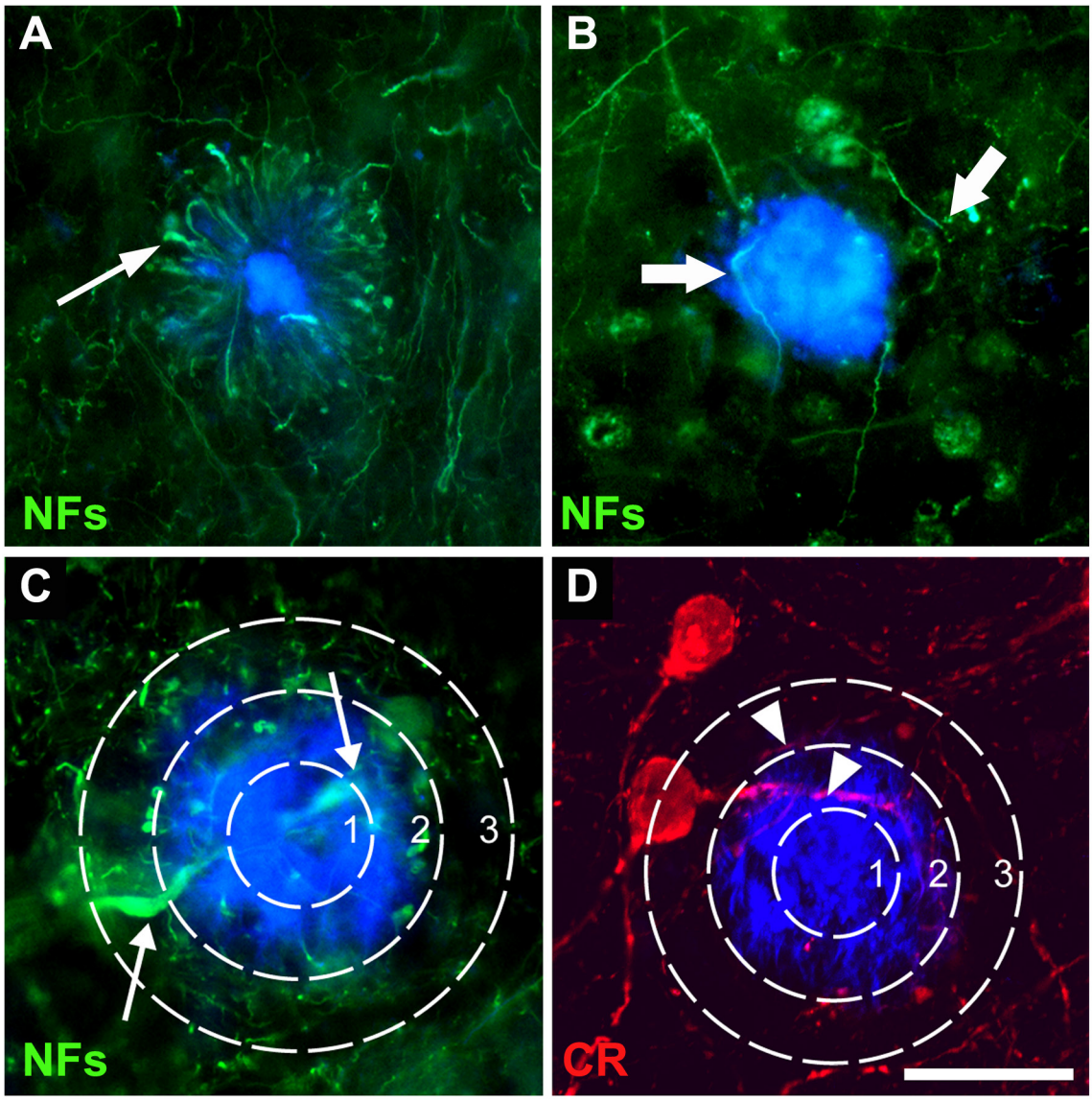
To minimize inter-sample variability, all immunohistochemical procedures were performed identically as outlined in Chapter 2. Sections were blocked for two hours in 10% goat serum (Sigma-Aldrich, St Louis, MO), 0.3% Triton-X by volume in 0.01M PBS at room temperature, followed by overnight incubation with primary antibodies in blocking solution at 4°C. Human and mouse cortical sections were co-immunolabelled with rabbit anti-CR (1:2000 dilution, Millipore) and a cocktail of mouse SMI32 (non-phosphorylated) and SMI312 (phosphorylated neurofilament triplet protein, NF) (both at 1:1000, Covance) to visualize CR-ir and NF-ir subpopulations of interneurons and principal neurons, respectively (Sampson et al., 1997; Kirkcaldie et al., 2002). Sections were then counter-stained with 0.0125% thioflavine-S (Sigma-Aldrich, St Louis, MO) to visualize fibrillar Aβ plaques.

Analysis of neurite susceptibility

For both AD and mouse cortex, three sections per individual were used. In each section, ten plaques were chosen (n = 30 plaques per case/animal) to assess their effects on CR-ir and NF-ir neurites. Plaques were chosen at random in layers 2-4, which contain the majority of CR-ir interneuron perikarya and processes (Fonseca and Soriano, 1995; del Río and DeFelipe, 1997; Park et al. 2002) as well as the highest plaque load (Duyckaerts et al. 1986; Braak et al. 2011). Using previously described criteria for pathological cytoskeletal alterations (Knowles et al. 1999; Le et al. 2001; D'Amore et al. 2003), labelled neurites with focal increases in diameter such as swellings, highly-undulating trajectories or with a change in course greater than 90° from the original trajectory, were counted as 'dystrophic' (Figure 3.1). Conversely, labelled neurites that did not meet any of the dystrophic criteria were considered normal. For each plaque, the number of CR-ir and NF-ir neurites traversing the core (zone 1), edge (zone 2) and periphery (zone 3) (defined as 50%, 100% and 150% of plaque diameter; Figure 3.1 C, D) was determined to assess whether these regions differed in neuropil damage. More than 3000 intact and dystrophic neurites per case type were counted. The average plaque area for human AD (1290 ± 101μm^2) or transgenic mice (1070 ± 67μm^2) was used to define "pseudo-plaque" areas placed randomly in images of control tissue using the grid function in ImageJ, used as estimates of normal neurite density within similar cortical regions. The number of neurites per plaque zone was normalized to the mean plaque diameter for each case to allow comparisons of proportions between zones. The mean number of dystrophic and normal neurites in each plaque zone was expressed as a percentage of the mean control neurite number. Neurite density loss in

Figure 3.1

Double-immunoflourescence for NFs (SMI312 and SMI32) and thioflavine-S (A-C) or calretininin and thioflavine-S (D) with a superimposed template showing zone 1 (plaque-core), zone 2 (plaque-edge) and zone 3 (plaque-periphery). Small arrows (A, C) denote characteristic swellings in dystrophic neurites, large arrows (B) highlight increased neurite curvature and tortuosity, while arrowheads (D) show normal-appearing neurites. Scale bar = 20 μm


A
NFs
B
NFs
C
1
2
3
NFs
D
1
2
3
CR

AD and transgenic tissue relative to control values was estimated (neurite density loss = 100 – [normal neurites + dystrophic neurites]/Wt normal neurites %).

Image acquisition

Images were captured using a Leica DM LB2 fluorescence microscope with a cooled CCD Magnafire (Optronics) camera, as outlined in Chapter 2. All image analysis was performed using NIH ImageJ (version 1.45p) software.

Statistical analysis

Statistical analyses for comparisons of group means was conducted by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc tests or unpaired Student's t-tests using GraphPad Prism software (version 5.0b).

3.3 Results

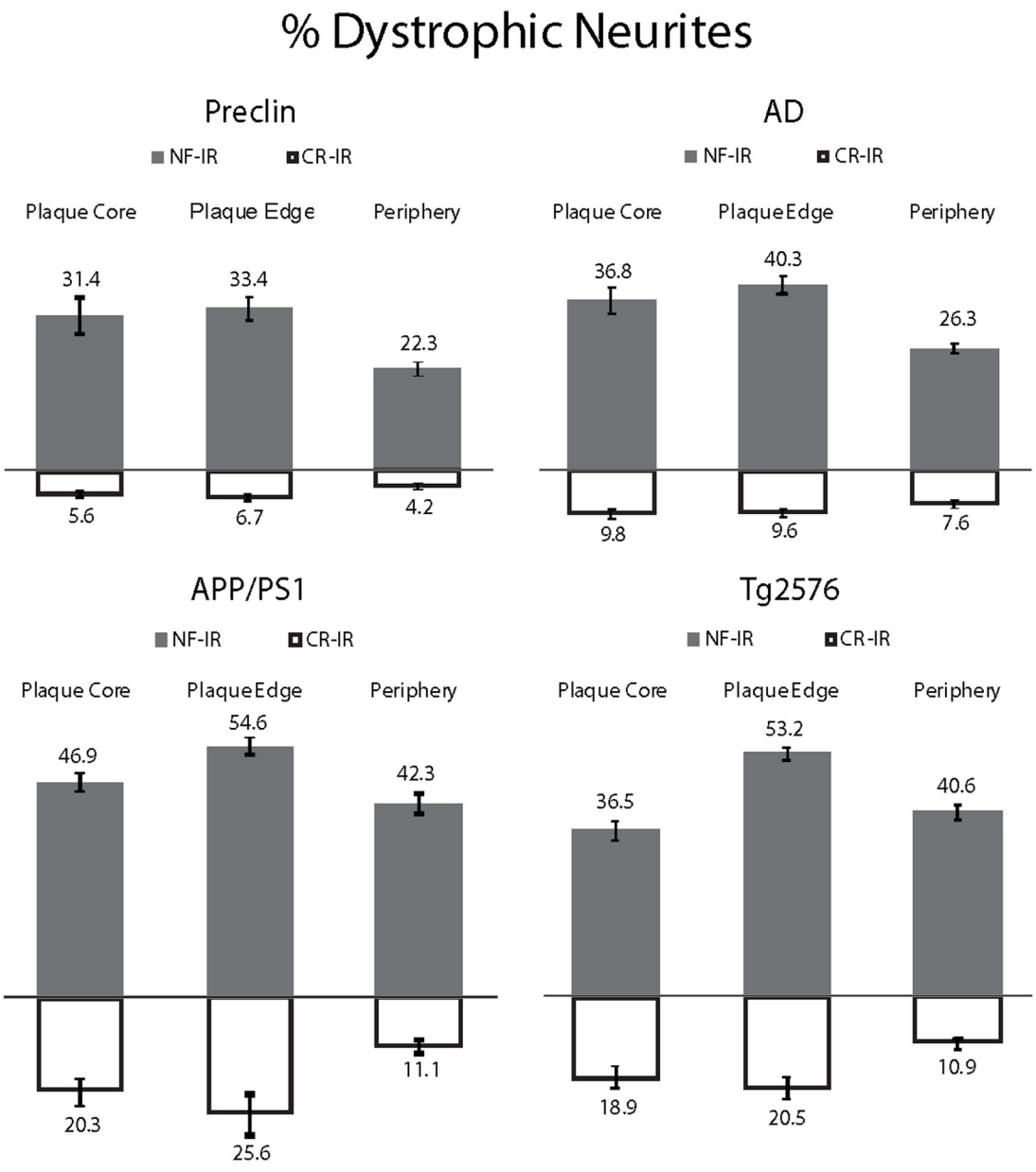
Calretinin-immunolabelled interneurons associated with Aβ plaques are more resistant to neuritic pathology than neurofilament-immunoreactive neurons

A cocktail of SMI32 and SMI312 antibodies was used to label the NF-ir subpopulation of neurites, chiefly deriving from pyramidal cells of cortical layers 2-6 (van der Gucht et al. 2007; Paulussen et al. 2011). In the rat neocortex, NF-ir pyramidal neurons account for approximately 10-13% of all neurons (Kirkcaldie et al. 2002), whereas 20-30% of neurons in human temporal cortex are NF-ir (Hof et al. 1990). Although a small proportion of PV-ir basket cells, as well as non-pyramidal neurites originating from subcortical and subthalamic regions may also be labeled by neurofilament antibodies, their fewer numbers and less extensive neurite fields ensure that pyramidal neurites make up the majority of NF-ir processes in cortical layers 2-6 (Thomson and Bannister 2003). Unlike CB and PV which are both expressed by subpopulations of pyramidal neurons (Hof and Morrison 1991; Jinno and Kosaka 2004), the neurofilament triplet is largely absent from CR-ir cells in layers 2-6 (DeFelipe 1997; Sampson et al. 1997).

The number of NF-ir and CR-ir neurites scored as ‘dystrophic’ or ‘normal’ were analysed at 50%, 100% and 150% of Aβ plaque diameters (zones 1, 2 and 3 respectively) and mean values were expressed as percentages of mean human and mouse control values. For all case types and zones analysed, there was a significantly higher percentage of dystrophic NF-ir, compared to CR-ir neurites (Figure 3.2). Of the three zones, NF-ir dystrophic neurites were most numerous at the plaque edge (zone 2) in APP/PS1 mice (54.6 ± 2.2%) followed by Tg2576 mice (53.2 ± 2.0%), sporadic AD (40.3 ± 1.7%) and preclinical AD cases (33.4 ± 1.9%; mean ± SEM, $p <$

Figure 3.2

Graphs illustrating the percentage of NF- (grey bars) and CR- (white bars) immunolabelled dystrophic neurites associated with fibrillar Aβ plaques at the three zones in AD, preclinical AD, APP/PS1 and Tg2576 transgenic mice respectively (± S.E.M.). There are significantly more dystrophic PR neurites traversing the Aβ plaque core, edge and periphery than CR dystrophic neurites within all case types ($p < 0.05$, Dunnett's post-hoc test).


% Dystrophic Neurites
Preclin
NF-IR
CR-IR
Plaque Core
Plaque Edge
Periphery
31.4
33.4
22.3
5.6
6.7
4.2
AD
NF-IR
CR-IR
Plaque Core
Plaque Edge
Periphery
36.8
40.3
26.3
9.8
9.6
7.6
APP/PS1
NF-IR
CR-IR
Plaque Core
Plaque Edge
Periphery
46.9
54.6
42.3
20.3
25.6
11.1
Tg2576
NF-IR
CR-IR
Plaque Core
Plaque Edge
Periphery
36.5
53.2
40.6
18.9
20.5
10.9

0.01). Similarly, CR-ir dystrophic neurites were most prevalent at the plaque edge in APP/PS1 (25.6 ± 2.5%), Tg2576 (20.5 ± 1.7%), sporadic (9.6 ± 0.8%) and preclinical (6.7 ± 0.7%) AD cases ($p < 0.01$). The greatest difference between NF-ir and CR-ir neurite responses in all tissue types was in the plaque periphery (zone 3) where there were, on average, four times as many dystrophic NF-ir neurites as CR-ir neurites (Fig. 3.2). Representative immunolabelled sections demonstrate a much higher degree of dystrophic changes in NF-ir neurites (Figure 3.3) than in CR-ir neurites (Figure 3.4).

Next, the number of neurites with normal morphology around Aβ fibrillar deposits was compared to controls, allowing for an estimate of the neurite density loss. Both NF-ir and CR-ir neurites exhibited comparable pronounced neurite density loss in the plaque core in AD, preclinical AD, APP/PS1 and Tg2576 cases (Figure 3.5; $p < 0.01$). However, no significant loss of CR-ir neurites was seen at the plaque edge or periphery in all cases ($p > 0.05$), in contrast to NF-ir neurites, which showed significant losses in all regions except for the plaque periphery in preclinical AD cases (Figure 3.5). Furthermore, in all four case types, the percentage of normal CR-ir neurites at the plaque edge (Figure 3.4 C, F, I, L) was significantly higher than for NF-ir neurites (Figure 3.3 C, F, I, L; $p < 0.01$). An interesting observation was that many normal CR-ir neurites in zone 3 elaborated processes around Aβ plaques, instead of traversing the plaque or stopping at the plaque border as NF-ir neurites often did (Figure 3.4).

The number of NF-ir dystrophic neurites is dependent on Aβ plaque size

The mean Aβ plaque size was determined in all of the case types, the largest plaques occurring in human AD cases (1290 ± 101μm^2) and the smallest in preclinical AD

Figure 3.3

Double immunofluorescence labeling of representative thioflavine-S plaques and NF-ir neurites from preclinical AD (A, B), end-stage AD (D, E), APP/PS1 (G, H) and Tg2576 (J, K) transgenic mice shows classical bulb-like swellings containing neurofilaments indicated by filled arrowheads in zone 1, small arrows in zone 2 and large arrows in zone 3. Normal-appearing neurites in association with thioflavine-S labeled fibrillar plaques are indicated by unfilled arrows. A large proportion of NF-ir neurites associated with Aβ plaques are dystrophic (blue fraction: C, F, I, L) and there is extensive neruite loss in end-stage AD and Tg2576 mice (grey fraction: F, L).

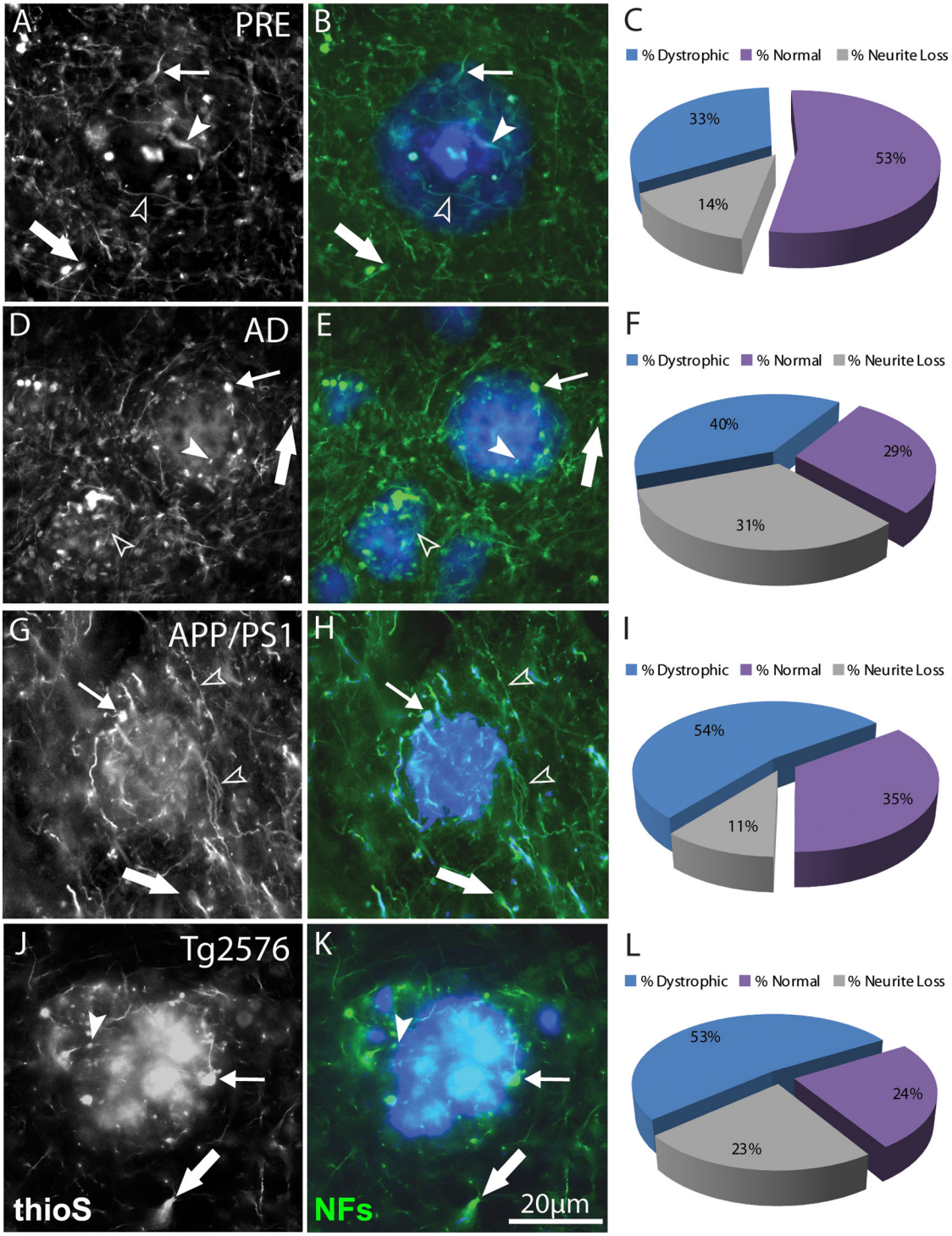

A
PRE
B
C
% Dystrophic
% Normal
% Neurite Loss
33%
53%
14%
D
AD
E
F
% Dystrophic
% Normal
% Neurite Loss
40%
29%
31%
G
APP/PS1
H
I
% Dystrophic
% Normal
% Neurite Loss
54%
35%
11%
J
Tg2576
K
L
% Dystrophic
% Normal
% Neurite Loss
53%
24%
23%
thioS
NFs
20µm

Figure 3.4

Double immunofluorescence labeling of representative thioflavine-S plaques and CR-ir neurites from preclinical AD (A, B), end-stage AD (D, E), APP/PS1 (G, H) and Tg2576 (J, K) transgenic mice shows a relative paucity of swellings containing calretinin (arrows). Filled arrowheads indicate normal-appearing CR neurites in association with thioflavine-S labeled fibrillar plaques. In most cases these neurites appear to elaborate around plaques. A large proportion of CR-ir neurites associated with Aβ plaques are normal (purple fraction: C, F, I, L) in all case types and there are comparatively fewer dystrophic neurites (blue fraction: C, F, I, L).

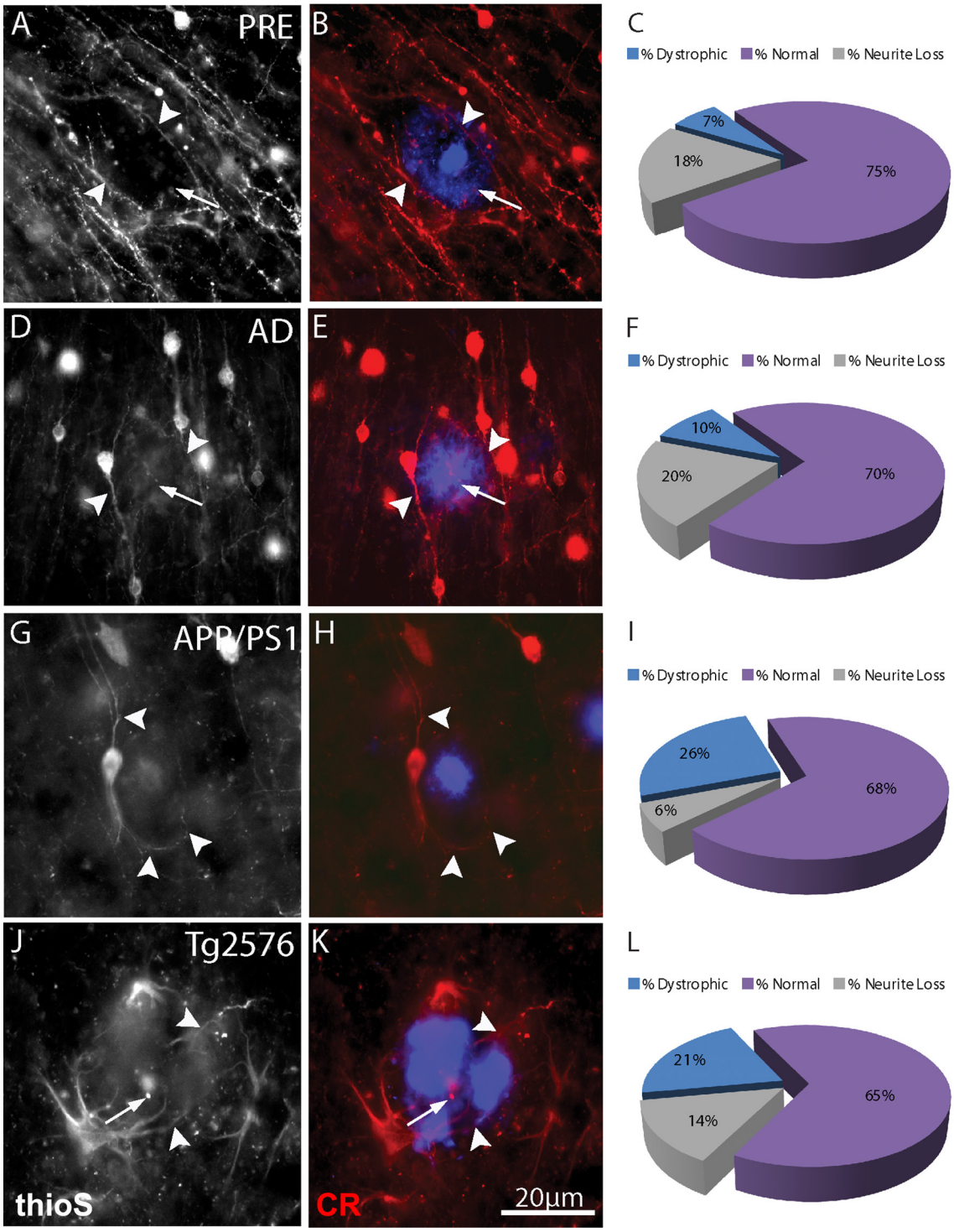
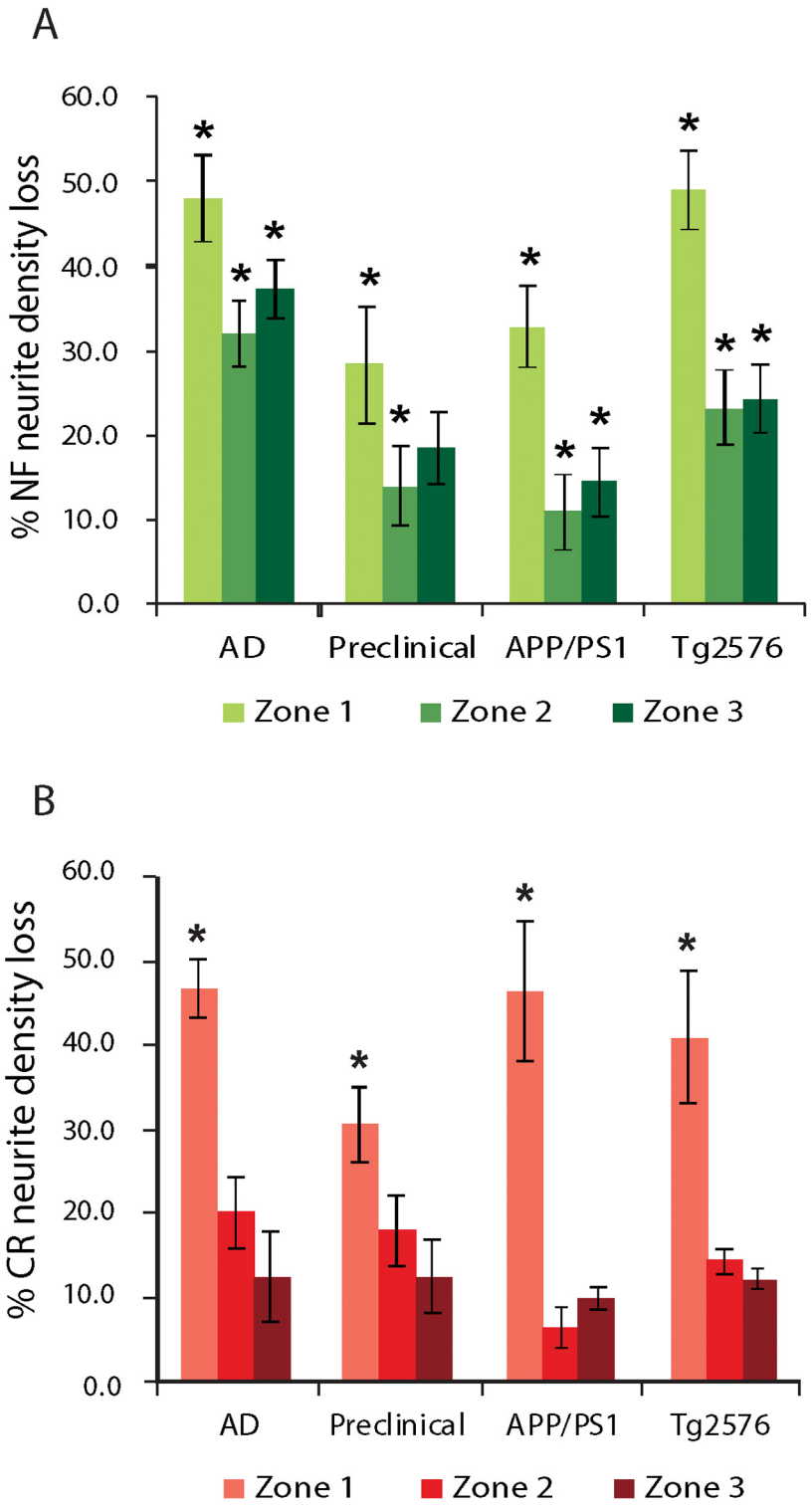

A
PRE
B
C
% Dystrophic
% Normal
% Neurite Loss
7%
18%
75%
D
AD
E
F
% Dystrophic
% Normal
% Neurite Loss
10%
20%
70%
G
APP/PS1
H
I
% Dystrophic
% Normal
% Neurite Loss
26%
6%
68%
J
Tg2576
K
L
% Dystrophic
% Normal
% Neurite Loss
21%
14%
65%
thioS
CR
20µm

Figure 3.5

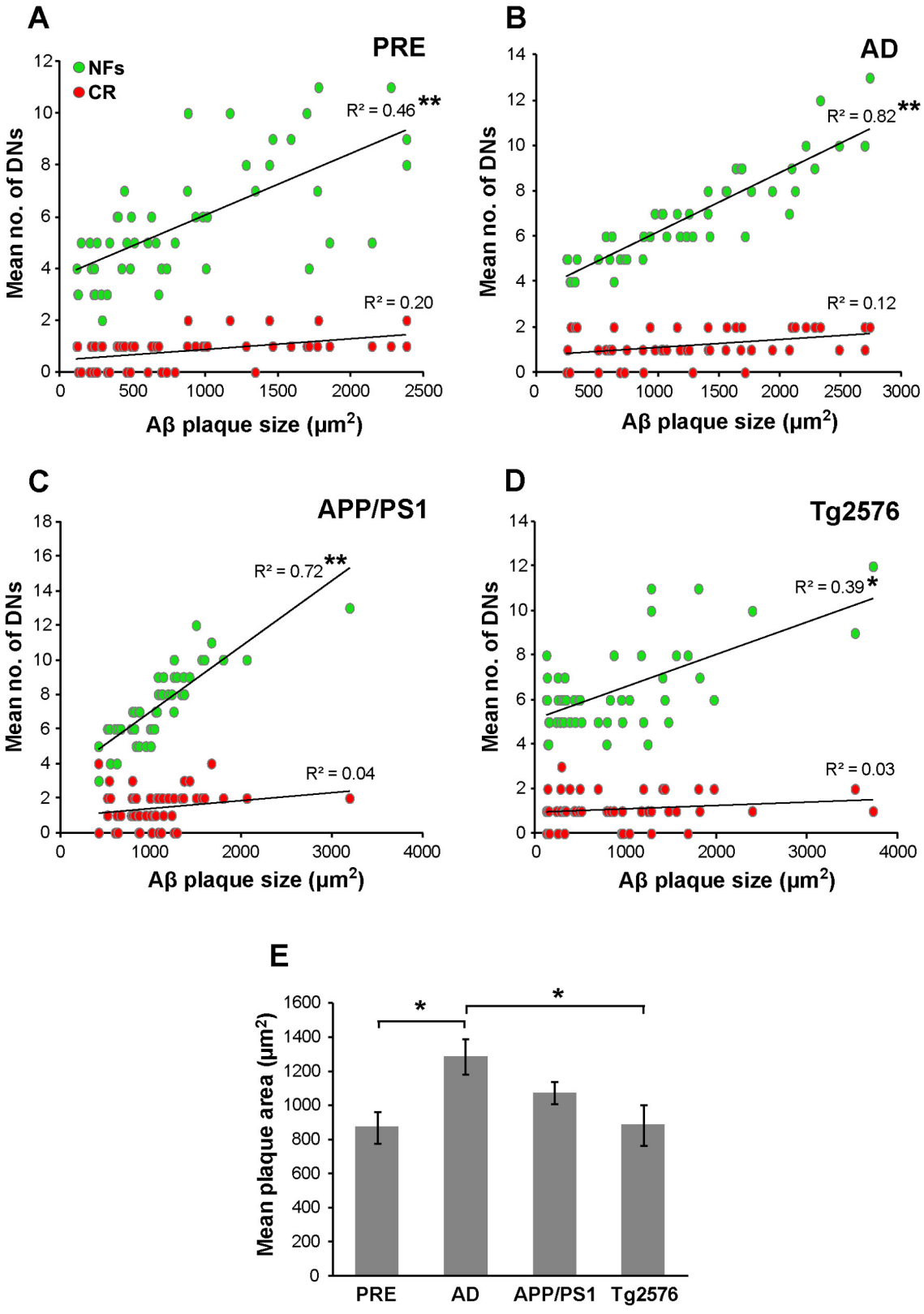
Graphs showing the percentage neurite density loss fro NF-ir (A) and CR-ir (B) neurites. There is a significant NF-ir neurite density loss for the different case types across all three regions (A, * $p < 0.01$, ANOVA), whereas CR-ir neurite density loss for the different case types was significant only in zone 1 (B, * $p < 0.01$, ANOVA). Error bars denote standard error of the mean.


A
% NF neurite density loss
60.0
50.0
40.0
30.0
20.0
10.0
0.0
AD
Preclinical
APP/PS1
Tg2576
Zone 1
Zone 2
Zone 3
B
% CR neurite density loss
60.0
50.0
40.0
30.0
20.0
10.0
0.0
AD
Preclinical
APP/PS1
Tg2576
Zone 1
Zone 2
Zone 3

cases (870 ± 93μm^2). In human AD cases, the mean number of dystrophic NF-ir neurites correlated very well with Aβ plaque size in both preclinical (r =.46, $p < 0.001$) and end-stage AD (r = .82, $p < 0.001$) cases (Figure 3.6). There was a similar pattern in TG mice, with higher numbers of NF-ir DNs associated with larger plaques in APP/PS1 (r =.72, $p < .001$, mean plaque size = 1072 ± 67μm^2) and Tg2576 (r =.39, $p < 0.01$, mean plaque size = 882 ± 118μm^2) animals. In contrast, the number of CR-ir DNs did not correlate significantly with Aβ plaque size in any of the case types examined (Figure 3.6).

Figure 3.6

The mean number of NF-ir, but not CR-ir, dystrophic neurites (DN) was significantly related to Aβ plaque size in both preclinical (A, **$p < 0.001$) and end-stage AD (B), as well as in APP/PS1 (C) and Tg2576 (D, *$p < 0.01$) transgenic mice. End-stage AD cases had the largest mean Aβ plaque size (E, $p < 0.05$). Error bars denote standard error of the mean.


A
PRE
NFs
CR
R² = 0.46**
R² = 0.20
Mean no. of DNs
Aβ plaque size (μm²)
B
AD
R² = 0.82**
R² = 0.12
Mean no. of DNs
Aβ plaque size (μm²)
C
APP/PS1
R² = 0.72**
R² = 0.04
Mean no. of DNs
Aβ plaque size (μm²)
D
Tg2576
R² = 0.39*
R² = 0.03
Mean no. of DNs
Aβ plaque size (μm²)
E
Mean plaque area (μm²)
*
*
PRE
AD
APP/PS1
Tg2576

3.4 Discussion

Previous studies in post-mortem AD tissue have reported limited or non-significant reductions in cortical interneuron subpopulations, with the exception of the loss of small subpopulations of NF-ir parvalbumin (PV) and calbindin (CB) interneurons (Hof and Morrison 1991; Hof et al. 1991, 1993; Fonseca and Soriano 1995; Sampson et al. 1997; Leuba et al. 1998; see Table 1.1 for more detail). This has led some researchers to conclude that interneurons are spared relative to pyramidal neurons even in advanced stages of AD (Davies 1998; Bell et al. 2006). Although reductions in interneuron numbers in the human hippocampus and entorhinal cortex are slightly more pronounced, there is profound early pyramidal neuron loss in these areas, which could possibly also affect interneurons (Solodkin et al. 1996). Therefore, interneuron loss appears to be secondary to pyramidal cell loss, particularly in layer 2 of the entorhinal cortex (Solodkin et al. 1996) and the dentate gyrus of the hippocampus (Brady and Mufson 1997).

Recently, this view has been challenged by reports of significant early reductions in hippocampal subpopulations of CB-ir (Koliastos et al. 2006), PV-ir (Popovic et al. 2008; Loreth et al. 2012) and CR-ir interneurons (Baglietto-Vargas et al. 2010; Takahashi et al. 2010) in APP/PS1 mice. As these mice overexpress fAD-associated mutant human APP and PS1, they develop a much accelerated AD pathology, with cognitive symptoms becoming apparent by 3-4 months of age (Garcia-Alloza et al. 2006a). However, most of these reports used cell death to indicate interneuron susceptibility, whereas it is now clear that network dysfunction occurs well before neuron death in human AD, and particularly in transgenic AD models which often do not have overt cell loss. Therefore, a comprehensive morphological analysis of

cortical CR-ir and NF-ir neurites associated with fibrillar Aβ plaques in early and end-stage human AD cases, as well as Tg2576 and APP/PS1 mice was conducted.

I found a progression of dystrophic changes in NF-ir neurites from early to end-stage human AD, which was closely mimicked in both APP/PS1 and Tg2576 transgenic mice lines. There was an increase in the number of dystrophic NF-ir neurites across all three case types, most prominent at the plaque periphery, followed by the surrounding neuropil and the plaque core, respectively (Figure 3.2). Concomitantly, the percentage of normal neurites relative to age-matched controls across these three regions decreased markedly, due to a general loss of neurite density in zones 1 and 2 (Figure 3.5). This is in agreement with previous work showing reduced axonal density and total length in AD plaque-associated neuropil (Dewitt and Silva 1996). The most severe changes occurred in end-stage AD, followed by the Tg2576 and APP/PS1 mouse models, and were least prominent in preclinical human AD cases. The finding that Aβ plaques exert a neurite damaging effect more distantly (i.e. zone 3) concurs with previous studies (D'Amore et al. 2003). Retrograde swellings in axons have been reported in sites several hundred microns from plaques *in vivo* (Vickers et al. 1996; Adalbert et al. 2009). This implies that the dystrophic changes are propagated to distal parts of neurites, or that plaques possess a 'halo' of toxicity that extends more distally (Koffie et al. 2009).

In contrast, dystrophic neurites constituted a smaller proportion of total CR-ir neurites in all three zones as compared to NF-ir neurites. Therefore, there were significantly more normal-appearing CR-ir neurites in zones 2 and 3. Less calretinin-labelled neurites, both normal and dystrophic, were observed in zone 1 relative to the other two zones examined. Contrary to NF-IR neurites, which tended to traverse

plaque cores, often showing signs of dystrophy, CR-ir neurites tended to either terminate at the plaque periphery, or much more commonly, to elaborate around it (Figure 3.4). Furthermore, unlike NF-ir DNs, the mean number of CR-ir DNs did not correlate with plaque size (Figure 3.6). In addition to their potential focal toxicity, larger plaques could also exert a 'physical' effect on the surrounding neuropil and cause structural damage to neurites like increased tortuosity (Knowles et al. 1999; Le et al. 2001; Woodhouse et al. 2005). Therefore, CR-ir neurites may be more resistant to 'physical' damage mediated by Aβ plaques.

One potential explanation for the relative preservation of CR-ir neurites shown here is that interneurons may have a higher capacity for structural plasticity in the normal and injured cortex. Recently, CR-ir interneurons were shown to exhibit greater structural plasticity in response to focal brain injury by rearranging their dendritic processes away from the injury site and into the unaffected neuropil (Blizzard et al. 2011). Furthermore, GABA-positive interneurons are relatively spared after focal ischemic injury (Frahm et al. 2004) and are able to extend new axons following proximal axotomy, suggesting a greater capacity for neurite regeneration (Fenrich et al. 2007). Long-term *in vivo* imaging studies in adult murine cortex show that, in contrast to pyramidal neurons which are highly stable under normal conditions, inhibitory neurons are much more labile, extending new dendritic arbors and displaying a higher degree of plasticity in existing branches (Lee et al. 2006, 2008). Taken together, these studies suggest that interneurons may have an increased propensity for structural remodelling in normal function or in response to focal damage such as Aβ plaques. In this regard, the results of the present study suggest that fewer CR-ir neurites traverse plaque cores (zone 1), while many elaborate around plaques (Figure 3.4), potentially signifying dynamic restructuring away from

these localized sites of neuropil injury, as previously described (Blizzard et al. 2011). Alternatively, the relative resistance of CR-ir neurites to Aβ plaque damage could reflect increased repair and regeneration following injury.

In addition to their higher capacity for structural remodelling, interneurons also express high levels of calcium-buffering proteins and may thus be better equipped to counter focal Aβ plaque-mediated calcium dysregulation reported in nearby neurons (Busche et al. 2008) and neurites (Kuchibhotla et al. 2008). Elevated levels of calcium could lead to distortion of neurite morphology and loss of dendritic spines, possibly by unregulated calcineurin activation (Wu et al. 2010). In this regard, Greene and colleagues (2001) suggest that calcium-buffering proteins like calbindin are upregulated in some pyramidal cell populations during normal ageing, as a potentially neuroprotective mechanism. For example, age-related decreases in calbindin could account for the loss of basal forebrain cholinergic neurons in AD, since the subpopulation of neurons retaining high levels of calbindin did not develop neurofibrillary tangles or degenerate (Riascos et al. 2011).

As noted earlier (see Section 1.4.3), the use of calcium-binding protein content to segregate subtypes of interneurons yields three main populations with various degrees of overlap (DeFelipe 1997; Markram et al. 2004). Parvalbumin-ir interneurons give rise to large- and small-sized basket cells, as well as chandelier cells, the former targeting proximal dendrites and somata while the latter specifically synapsing on the axon initial segment. Calretinin-ir interneurons predominantly comprise double bouquet, bipolar, bitufted and Martinotti cell morphologies and tend to synapse on distal and apical dendrites. There is very little overlap between the expression of these immunohistochemical markers as the cells that express them

perform very different roles in inhibition (Kawaguchi and Kubota 1997; Xu et al. 2010). Calbindin-ir interneurons on the other hand,

In conclusion, this study showed that CR-ir interneurons are less susceptible to cytoskeletal pathology mediated by Aβ plaques than NF-ir principal neurons in human AD cases and transgenic mouse models. It is likely that a combination of factors, such as a higher capacity for structural remodelling and regeneration following focal injury, contribute to this relative resistance. Moreover, recent studies suggest that a high content of calcium-binding proteins could be neuroprotective in disorders such as AD. Finally, this study contributes to previous work showing that NF-content may predispose the subpopulations of neurons that express them to structural damage in AD (Hof et al. 1993; Vickers et al., 1996, 1997, 2000; Sampson et al. 1997).

4. Synaptic remodelling in Alzheimer's disease and transgenic mouse models

4.1 Introduction

The evolving symptomology of Alzheimer's disease (AD) is linked to a pattern of neuronal degeneration that progressively spreads throughout the brain, affecting principally synaptic connections within and to the cerebral cortex. Neurofibrillary tangle formation leading to overt nerve cell death is a lengthy process, likely taking months to years whereas significant cognitive deficits may manifest long before significant cell loss is observed (Scheff et al. 2006). In addition, most transgenic murine AD models that include Aβ plaque formation show little to no cell loss even with severe cognitive dysfunction (reviewed in Crews et al. 2010), adding to the proposal that cognitive decline in AD may be predominantly mediated by functional disruption, including synaptic degeneration, rather than overt nerve cell degeneration.

Synapse loss has been reported to occur very early in AD-affected brain regions such as frontal or temporal cortex, and may correlate more closely with cognitive decline than plaques or tangles (Terry et al. 1991; Scheff et al. 2006). Nonetheless, the loss of synapses and dendritic spines near fibrillar Aβ plaques (Knafo et al. 2009; Koffie et al. 2012), and a reduction in experience-induced expression of plasticity-related proteins Arc and c-Fos in transgenic AD mice, suggest that plaques induce functional deficits at existing synapses (Palop et al. 2007; Rudinskiy et al. 2012). Network activity may be further disrupted by signalling imbalances: it has been suggested that AD progresses in a neurotransmitter-specific manner, with cholinergic synapses degenerating early, followed by glutamatergic terminals, leaving inhibitory γ-

aminobutyric acid (GABA) signalling largely unaffected even in advanced AD cases (Bell et al. 2006). Post-mortem studies in end-stage AD tissue have shown a relative sparing of cortical GABAergic presynaptic sites compared to cholinergic or glutamatergic markers (Bell et al. 2006, 2007), unaltered postsynaptic $GABA_A$ receptor density and protein levels (Rissman and Mobley 2011), as well as limited decreases in cortical GABA concentrations (Lowe et al. 1988; Siedl et al. 2001). Similarly, studies in aged AD transgenic mouse models have shown neurotransmitter-specific decreases in presynaptic markers (Bell et al. 2003; Cassano et al. 2012), reinforcing their usefulness as models of synaptic pathology in AD.

Therefore, human preclinical and end-stage human AD cases as well as transgenic APP/PS1 mice were used to determine cortical excitatory and inhibitory synaptic density and protein levels, compared to healthy age-matched control cases and wildtype littermates respectively. By immunolabelling for vesicular glutamate transporter 1 (VGlut1) and vesicular GABA transporter (VGAT), excitatory and inhibitory presynaptic bouton density and mean size in neuropil within and surrounding Aβ deposits, as well as in plaque-free regions were analyzed and compared to similar cortical regions in controls. Additionally, APP/PS1 mice crossed with YFP-expressing mice were used to analyse perisomatic inhibitory synapse numbers in Aβ plaque-rich neuropil, to determine if neurons closer to Aβ plaques are selectively disinhibited compared to those further away.

4.2 Materials and Methods

Human tissue

Human brain tissue included six sporadic end-stage AD and six preclinical AD cases, as well as five age-matched non-demented control cases lacking Aβ plaques or neurofibrillary pathology (see Chapter 2 for details).

Mouse tissue

Twelve-month-old APP/PS1 (APP_{Swe}×$PS1_{M146L}$) (Borchelt et al. 1997) and age-matched wildtype mice (*n*=5, for each strain) were used for synaptic marker analysis (see Chpater 2 for details). For the perisomatic synaptic labelling experiments, Tg(Thy1-YFP)16Jrs/Jtg/tg males (Feng et al. 2000; Jax Laboratories) were crossed with female APP/PS1tg/wt mice (both on C57/Bl6 background strain) and the resulting APP/PS1tg/wt YFPtg/wt and APP/PS1wt/wt YFPtg/wt mice were sacrificed at 2 or 12 months of age, as described above. Tissue was post-fixed for 2h at 4°C, before serial 40µm coronal sections were cut on a vibrating microtome from bregma–1.06mm to bregma–2.54mm (Franklin and Paxinos, 2007), and immunolabelled as outlined below.

Immunohistochemistry

To minimize inter-sample variability, all immunohistochemical procedures were performed identically. Sections were blocked for two hours in 10% goat serum, 0.3% Triton-X by volume in 0.01M PBS at room temperature, followed by overnight incubation with primary antibodies in blocking solution at 4°C. Alternate human and mouse cortical sections were immunolabelled with rabbit anti-VGlut-1 and VGAT (both 1:500, Synaptic Systems). A subset of mouse cortical sections were also

immunolabelled with mouse anti-GAD67 (1:1000, Millipore), mouse anti-GAD65 (1:200, Millipore) and rabbit anti-synaptophysin (1:200, Millipore). The tissue was then counter-stained with 0.125% Thioflavine-S (Sigma-Aldrich) in 60% 0.01M PBS and 40% ethanol for three minutes, followed by extensive washes in 0.01M PBS to stain fibrillar Aβ plaques. Thioflavine-S stains a subset of β-amyloid deposits with a fibrillar substructure, but not diffuse deposits (Dickson and Vickers, 2001). Alternate serial sections from YFP×APP/PS1 mice were labelled with mouse anti-GAD67 (1:1000, Millipore), rabbit anti-synaptophysin (1:200, Millipore) or mouse anti-GAD65 (1:200, Millipore). Primary antibody binding was visualized using species-specific fluorescent secondary antibodies conjugated to Alexa Fluors 488, 546 and 633 (1:500, Molecular Probes).

Immunoblotting

For Western blot analysis, whole brains were harvested and cortices were dissected from APP/PS1 and control animals at 2 and 12 months of age (n = 3 each, 3 repeats per sample) and immediately frozen in liquid nitrogen. Tissue was then homogenized in ice-cold SDS sample buffer (Appendix A) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). Following SDS-PAGE, membranes were incubated overnight in primary antibodies, including a combination of VGlut-1 (1:5000, Synaptic Systems), VGAT (1:2500, Synaptic Systems), synaptophysin (1:5000, Millipore) and β-actin (1:5000, Sigma-Aldrich). Membranes were washed in Tris-buffered saline with 0.1% Tween-20. Species-appropriate horseradish peroxidase conjugated secondary antibodies (1:2000, DAKO) were applied and visualized with a chemiluminescent peroxidase substrate kit (Millipore).

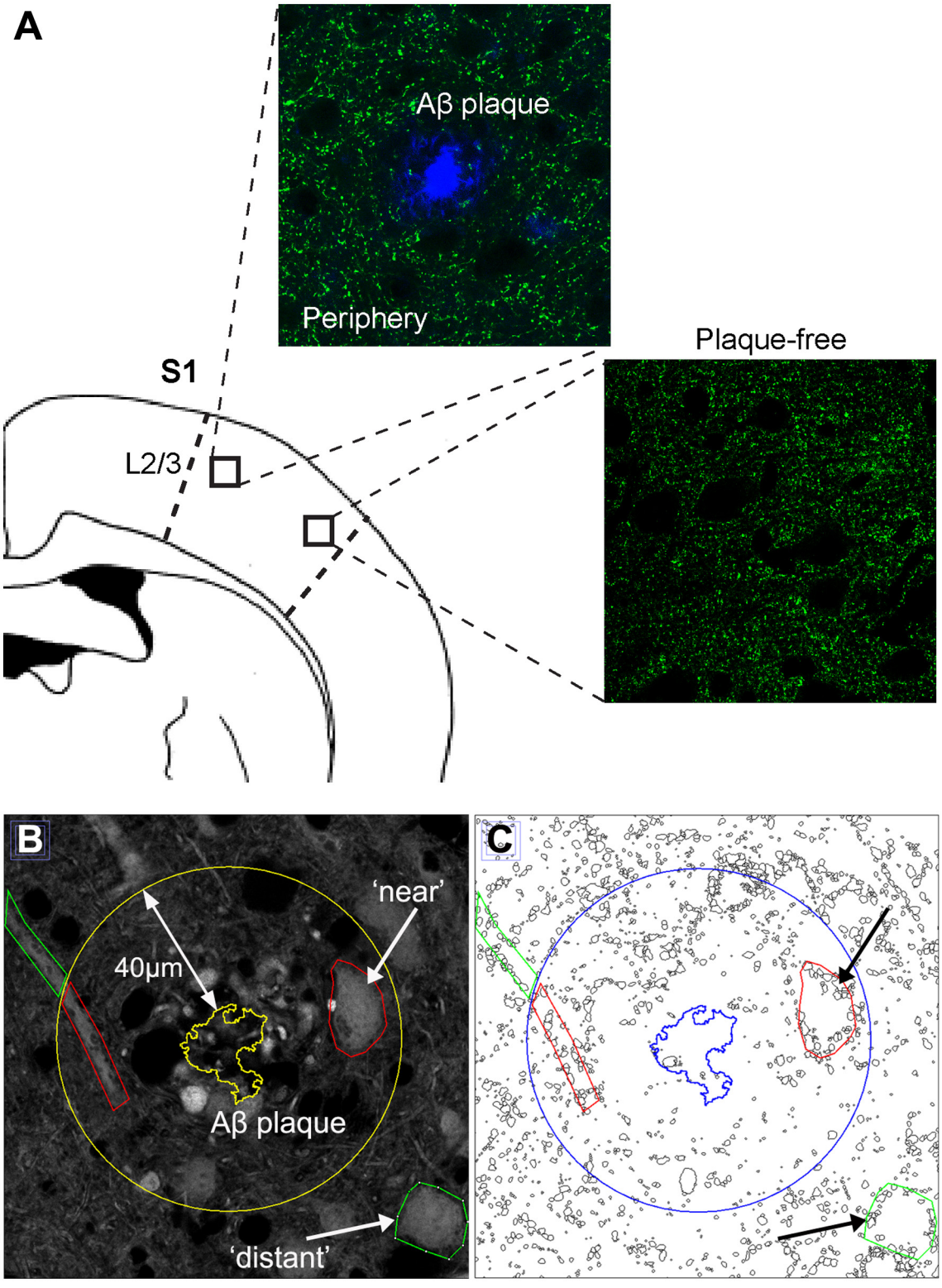
Image acquisition and analysis of presynaptic vulnerability

Ten plaque-rich and plaque-free regions in layers 2-4 of human temporal cortex and layer 2/3 of mouse somatosensory (S1) cortex (Figure 4.1A), as well as equal-sized regions in control cases and animals, were chosen for analysis of bouton density and size. Regions of interest were defined around ThioS counter-stained Aβ deposits (plaque area ≥ 500μm^2) that were at least 40μm from the edge of the plaque. These regions were defined as the plaque-periphery. Similarly, regions of interest in plaque-free neuropil, as well as within plaques were selected for analysis. Images were automatically thresholded and individual puncta were segmented with an ImageJ watershed algorithm (http://bigwww.epfl.ch/sage/soft/watershed) after application of Gaussian blurring (σ=1) as previously described (Sweet el al. 2010). Particles ranging from 0.15–2.0 μm^2 were quantified (Chattopadhyaya et al. 2007) and total bouton number and mean punctum size for each synaptic marker were determined using appropriate stereological techniques. Bouton cross-sectional densities were defined as the total number of labelled boutons counted per area of analysis and mean densities were expressed as a percentage of controls.

APP/PS1tg/wt × Thy1-YFP transgenic mice and age-matched APP/PS1wt/wt Thy1-YFPtg/wt littermate controls (at 2 and 12 months old, n=5 each) were used for perisomatic puncta analysis. Ten plaque-rich and plaque-free regions in layer 2/3 of somatosensory cortex per transgenic case, as well as equal-sized regions in control animals were chosen for analysis. Regions of interest were defined around the somata of YFP-positive pyramidal cells “near” (<40μm) or “distant” (>40μm) from the closest Aβ deposit (Figure 4.1B). These regions were defined as a 2μm zone adjacent to the soma, where the majority of cortical basket interneurons form

Figure 4.1

(A) Sections from layer 2/3 of somatosensory (S1) cortex in mice, and layers 2-4 of temporal cortex in human cases, were immunolabelled with presynaptic bouton markers. Bouton densities were calculated in areas corresponding to Aβ plaque, the plaque periphery and in plaque-free neuropil. To characterize presynaptic perisomatic boutons, YFP +/- soma that were near (< 40 μm) or distant (> 40 μm) from an Aβ plaque (B) were identified, followed by a watershed segmentation algorithm to isolate bouton puncta (C).


A
Aβ plaque
Periphery
Plaque-free
S1
L2/3
B
'near'
40μm
Aβ plaque
'distant'
C

synapses (Packer and Yuste 2011). Within this area GAD65-, GAD67- or synaptophysin (SYN)-ir puncta 0.15–2.0 μm^2 in size (Chattopadhyaya et al. 2004) were counted automatically, using ImageJ, to determine the number of boutons per pyramidal cell soma (Figure 4.1C). These counts were averaged across all pyramidal neurons measured, for each animal. Similarly, total bouton numbers for each marker were determined in plaque-free neuropil (area=17500μm^2), an area representing the plaque periphery (where $r_{periphery} - r_{plaque} \approx 40\mu m$) and within plaques. Bouton densities were defined as the total number of labelled boutons counted per area of analysis and mean densities were compared between transgenic animals and controls.

Confocal stacks (Z-step = 1.0µm, 3-5 sections per animal; image window size=17500μm^2) were acquired with a 63× oil-immersion objective (NA=1.4; Zeiss) using a confocal microscope (Zeiss LSM510) equipped with Zen software and Ar 488, HeNe 543 and HeNe 633 lasers. Scans from each channel were collected individually in multiple-track mode and subsequently merged. Care was taken to use the lowest laser power, and no bleed-through was visible between Alexa 488, Alexa 546 and Alexa 633 channels. Images were acquired using the same acquisition parameters for all samples and were saved as TIFF files for analysis with NIH ImageJ software.

Statistical analysis

Statistical analyses for comparisons of group means was conducted by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc tests or unpaired Student's t-tests using GraphPad Prism software (version 5.0b).

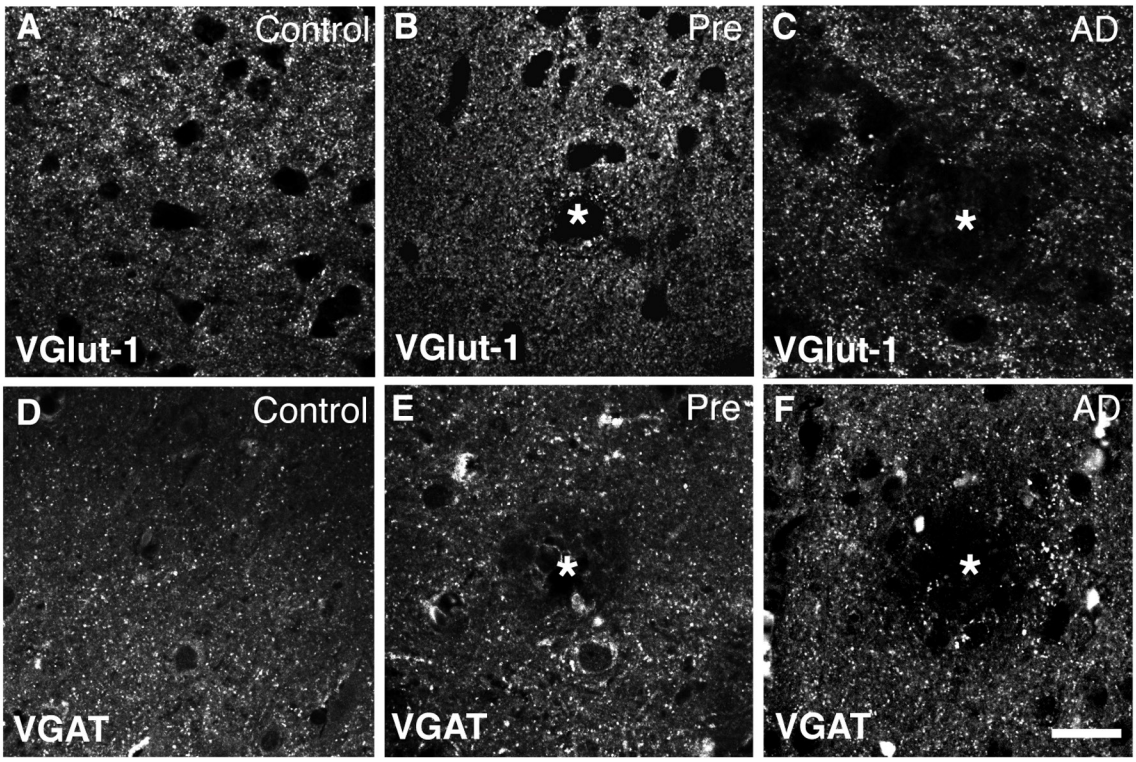
4.3 Results

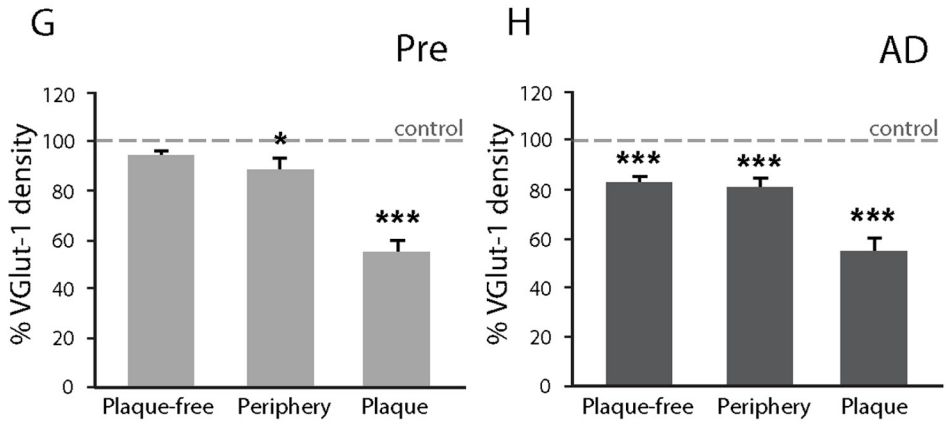
Differential effects of AD-related Aβ pathology on excitatory and inhibitory synaptic markers

Human preclinical AD, end-stage AD and APP/PS1 mouse cases, as well as corresponding age-matched controls, were immunolabelled with antibodies against either VGlut1, the main glutamate transporter found in excitatory synaptic vesicles in the cortex (Fremeau et al. 2001), or VGAT, the inhibitory equivalent (McIntire et al. 1997). The cross-sectional density of VGlut1-immunoreactive (VGlut1-ir) and VGAT-ir boutons around fibrillar Aβ plaques, in the surrounding neuropil and in plaque-free regions was determined and compared to similar regions in age-matched control cases. Within Aβ plaques, presynaptic marker bouton densities were significantly reduced in both human case types (preclinical 54.4 ± 5.1, end-stage 55.3 ± 5.1% of controls for VGlut1-ir boutons; preclinical 52.5 ± 6.0, end-stage 61.3 ± 3.9% of controls for VGAT-ir boutons; mean ± SEM; $p < 0.001$; Figure 4.2 B, E, G, I and C, F, H, J respectively), as well as in APP/PS1 mice (25.0 ± 6.7 and 11.4 ± 2.3% of VGlut1 and VGAT wildtype densities respectively, $p < 0.001$; Figure 4.3 B, C and E, F respectively). However, there was no significant decrease in VGAT-ir bouton density in the plaque periphery or in plaque-free neuropil in all three case types compared to controls (Figure 4.2 I, J and Figure 4.3 C, F). For VGlut1-ir boutons, end-stage AD cases showed a significant decrease in density in all three regions (Figure 4.2 H), whereas preclinical AD cases and APP/PS1 mice showed a significant decrease only in the Aβ plaque periphery. Interestingly, in preclinical AD cases, there was no significant change in VGlut-1 bouton density in plaque-free neuropil ($p = 0.07$), although APP/PS1 mice showed a significant increase in bouton density compared to wildtype ($p < 0.05$).

Figure 4.2

Representative immunofluorescence images of excitatory (A–C) and inhibitory (D–F) presynaptic bouton markers in human control, preclinical AD and end-stage AD cases showing a marked decrease in presynaptic density within Aβ plaques (represented by asterix). Graphs illustrating the relative bouton density (expressed as per cent of controls) of excitatory (G, H) and inhibitory (I, J) markers in preclinical and end-stage AD tissue (± S.E.M.) show that within all case types examined there was a significant loss of both excitatory and inhibitory presynaptic boutons within Aβ plaques. However, there was no loss of inhibitory terminals in the plaque periphery or in plaque-free neuropil of both preclinical (I) and end-stage AD (J) tissue (*$p < 0.05$; ***$p < 0.001$). Scale bar = 20 μm.


A
Control
VGlut-1
B
Pre
VGlut-1
C
AD
VGlut-1
D
Control
VGAT
E
Pre
VGAT
F
AD
VGAT


G
Pre
% VGlut-1 density
control
*
***
Plaque-free
Periphery
Plaque
H
AD
% VGlut-1 density
control
***
***
***
Plaque-free
Periphery
Plaque

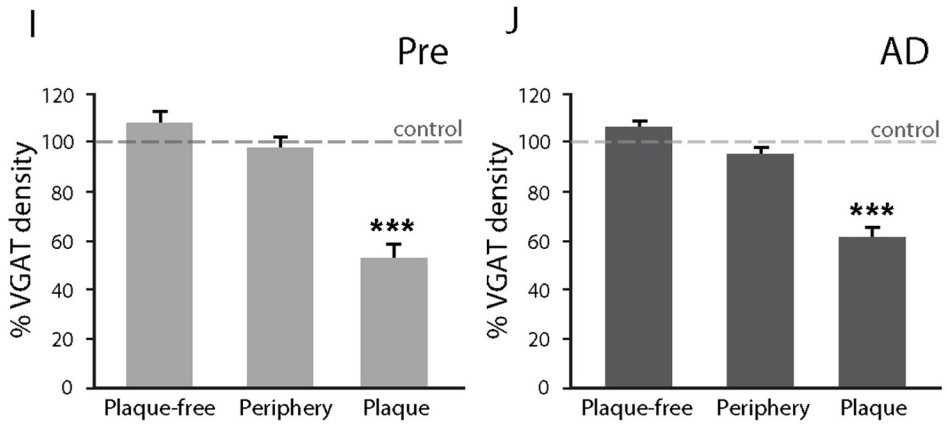
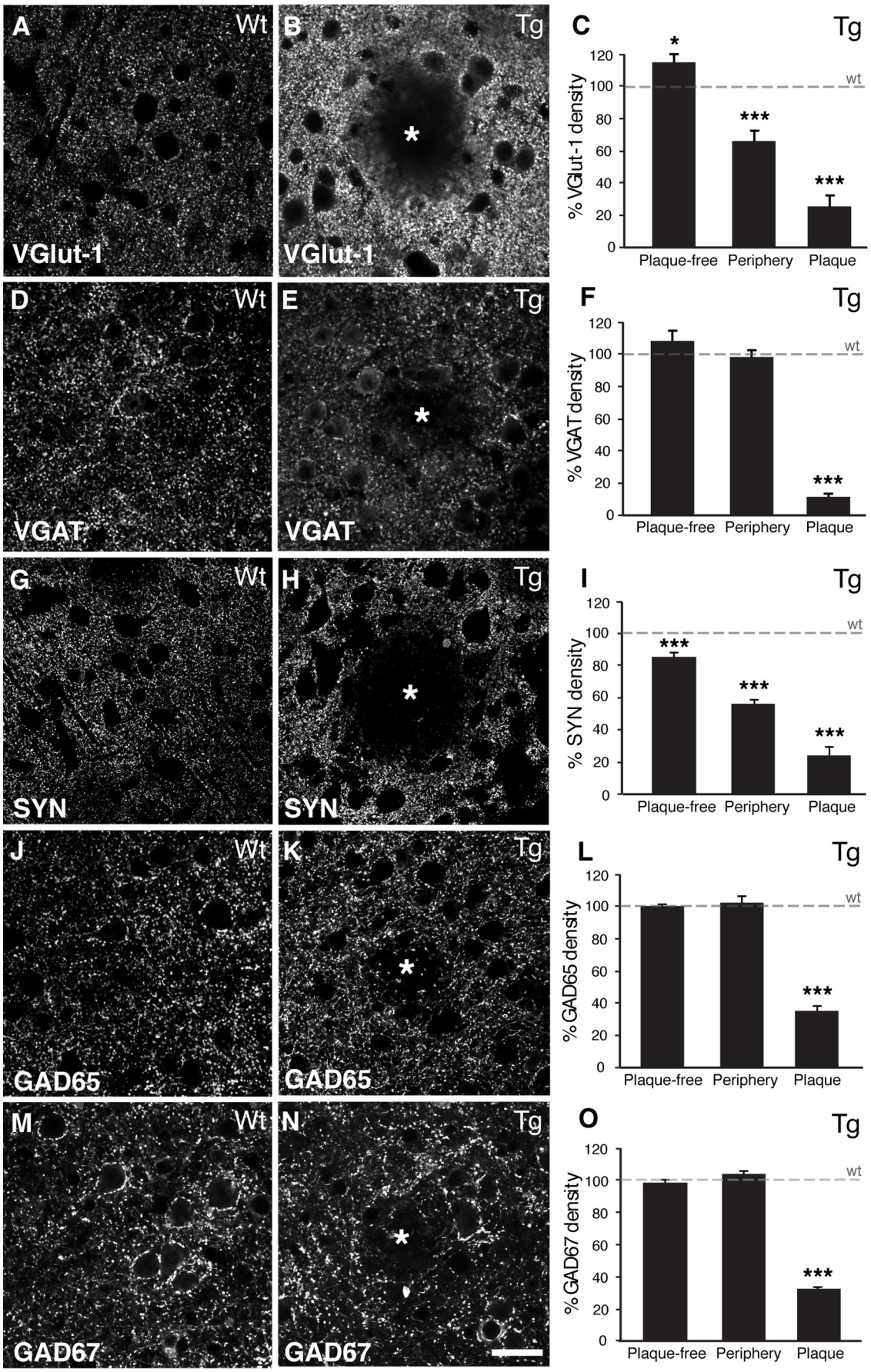

I
Pre
% VGAT density
control
***
Plaque-free
Periphery
Plaque
J
AD
% VGAT density
control
***
Plaque-free
Periphery
Plaque

Figure 4.3

Representative immunofluorescence images of excitatory (A, B), inhibitory (D, E) and pan-synaptic (G, H) presynaptic bouton markers, as well as the two isoforms of the GABA-synthesizing enzyme GAD65 (J, K) and GAD67 (M, N), in wildtype and transgenic mouse tissue showing a marked decrease in presynaptic density within Aβ plaques (represented by asterix). While all presynaptic markers were decreased inside Aβ plaques, only VGlut-1 (C) and SYN (I) density were significantly decreased in the plaque periphery or in plaque-free neuropil compared to controls (*$p < 0.05$; ***$p < 0.001$). Scale bar = 20 μm.


A
Wt
VGlut-1
B
Tg
VGlut-1
C
Tg
% VGlut-1 density
wt
Plaque-free
Periphery
Plaque
D
Wt
VGAT
E
Tg
VGAT
F
Tg
% VGAT density
G
Wt
SYN
H
Tg
SYN
I
Tg
% SYN density
J
Wt
GAD65
K
Tg
GAD65
L
Tg
% GAD65 density
M
Wt
GAD67
N
Tg
GAD67
O
Tg
% GAD67 density

To verify the identity of transporter-labelled structures, 12 month-old APP/PS1 tissue was labelled for GAD65 (Figure 4.3 J-L) and GAD67 (Figure 4.3 M-O), finding no significant difference in bouton density outside of Aβ plaques, whereas the pan-synaptic marker synaptophysin (Figure 4.3 G-I) showed significant reductions in all three regions examined (24.5 ± 5.0, 56.2 ± 2.5 and $85.3 \pm 3.1\%$ of control plaque, periphery or in plaque-free neuropil, respectively; $p < 0.001$). Subsets of human cases were also labelled with these markers, however consistent labelling for analysis was unable to be achieved.

Relative preservation of cortical VGAT protein, but decreased VGlut1 protein, levels in APP/PS1 mice

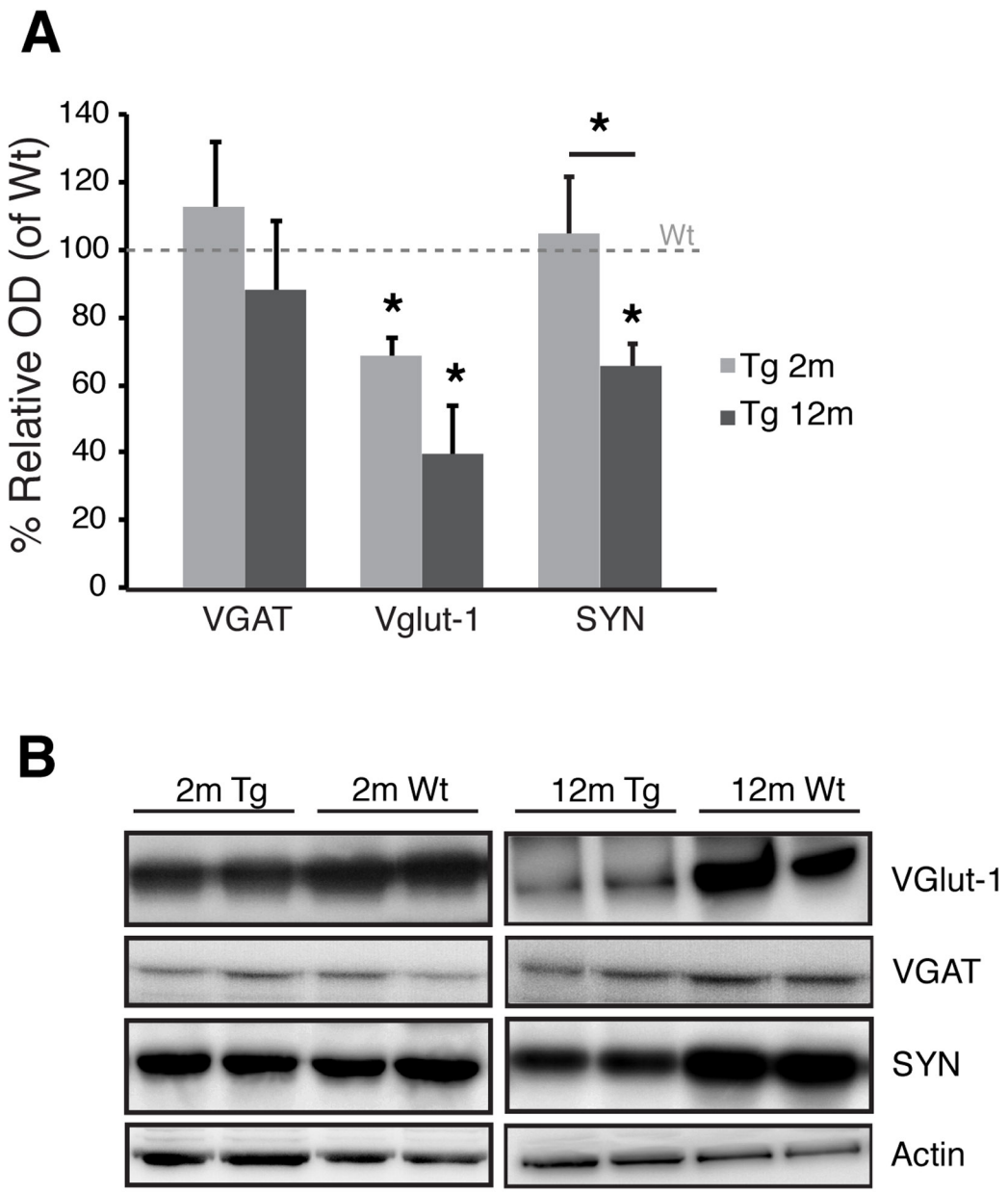
In addition to bouton metrics, VGlut1 and VGAT protein levels in APP/PS1 and wildtype mice were also determined. Cortical tissue from 2 and 12 month-old animals was extracted and aliquots were immunoblotted in triplicate. The relative abundance of these proteins compared to wildtype levels and representative Western blots reflecting protein levels in cortical samples at the two ages examined are shown in Figures 4.4 A and B, respectively. VGAT levels did not change with age or genotype ($p = 0.45$), but there was a significant reduction in both 2 and 12 month-old TG VGlut1 levels compared to littermate controls (68.3 ± 5.5 and $39.5 \pm 14.3\%$ of wildtype levels respectively, $p < 0.05$). There was also a reduction in synaptophysin protein levels in cortical synaptosomes from older TG mice compared to wildtype animals ($35.0 \pm 6.7\%$ reduction, $p < 0.05$).

Increase in GABAergic bouton size in Aβ plaque-rich neuropil

Photometric analysis in ImageJ also demonstrated a significant increase in the size of both VGlut1-ir and VGAT-ir boutons in the plaque-periphery and plaque-free

Figure 4.4

Immunoblots of cortical extracts from APP/PS1 animals showed a significant decrease in excitatory presynaptic marker levels compared to control tissue but no difference in inhibitory presynaptic protein marker levels (A, B) (*$p < 0.05$).


A
% Relative OD (of Wt)
140
120
100
80
60
40
20
0
Wt
*
*
*
*
VGAT
Vglut-1
SYN
Tg 2m
Tg 12m
B
2m Tg
2m Wt
12m Tg
12m Wt
VGlut-1
VGAT
SYN
Actin

neuropil of preclinical AD cases compared to controls (Figure 4.5 A, B). Interestingly, there was also an increase in VGAT-ir mean bouton size in the end-stage AD plaque-periphery, whereas there was a net decrease in VGlut1-ir bouton size (Figure 4.5 C, D). In TG mice, VGAT-ir bouton sizes were similar to littermate controls in plaque-free and plaque-adjacent tissue, but within Aβ plaques they were significantly reduced ($p < 0.001$; Figure 4.5 F). Similarly, TG VGlut1-ir boutons were smaller in Aβ plaques but larger in the plaque-periphery compared to littermate controls (Figure 4.5 E)

Pyramidal somata close to Aβ plaques have fewer perisomatic GAD67 but not GAD65 boutons

Perisomatic inhibition plays an important role in controlling the overall excitability of neurons and is thought to be crucial for rhythmic network activity (Packer and Yuste 2011). To determine the effect of plaque proximity on perisomatic input, YFP-positive pyramidal somata "near" (< 40μm) and "distant" (> 40μm) from Aβ deposits were analyzed. Overall, in APP/PS1 +/– cortex the mean number of SYN-ir and GAD67-ir boutons per soma decreased significantly compared to control littermates, irrespective of distance from plaque ($p < 0.01$). However, there was no loss of GAD65-ir perisomatic boutons on either near (19.8 ± 0.6, $n = 130$) or distant (21.8 ± 0.5, $n = 140$) somata compared to control mice (20.5 ± 0.5, $n = 250$; Figure 4.6 D). In contrast, there was a significant decrease in SYN-ir boutons on somata located near plaques (16.6 ± 0.9, $n = 190$ somata), compared with boutons on distant somata (25.2 ± 1.1, $n = 205$ soma, $p < 0.01$; Figure 4.6 D). There was no significant difference between GAD67-ir perisomatic boutons on near (13.9 ± 0.3, $n = 98$) versus distant (15.4 ± 0.3, $n = 181$; $p = 0.1$; Figure 4.6 D) somata, although both were significantly lower than in control animals (18.0 ± 0.3; $p < 0.01$). It was

Figure 4.5

Graphs illustrating the mean size of excitatory (A, C, E) and inhibitory (B, D, F) presynaptic boutons in preclinical AD, end-stage AD and transgenic mouse tissue compared to controls (± S.E.M.). Both excitatory (A) and inhibitory (B) presynaptic boutons were increased in size in the plaque-periphery and in plaque-free neuropil in preclinical AD cases, however only inhibitory bouton size was significantly increased in end-stage AD cases (C, D). Transgenic mouse cases showed a marked decrease in bouton size in both excitatory (E) and inhibitory (F) terminals only within Aβ plaques (ANOVA, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

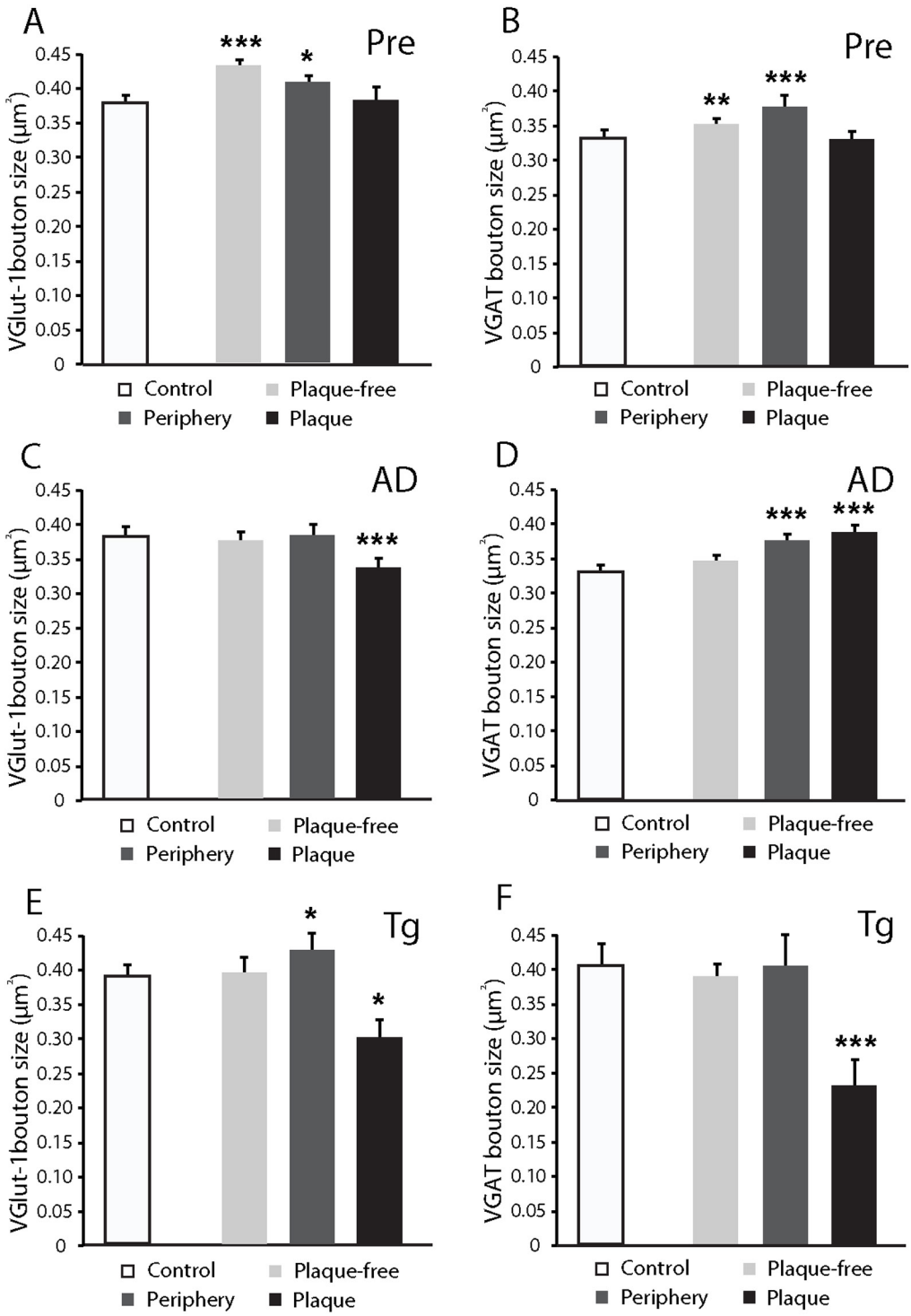
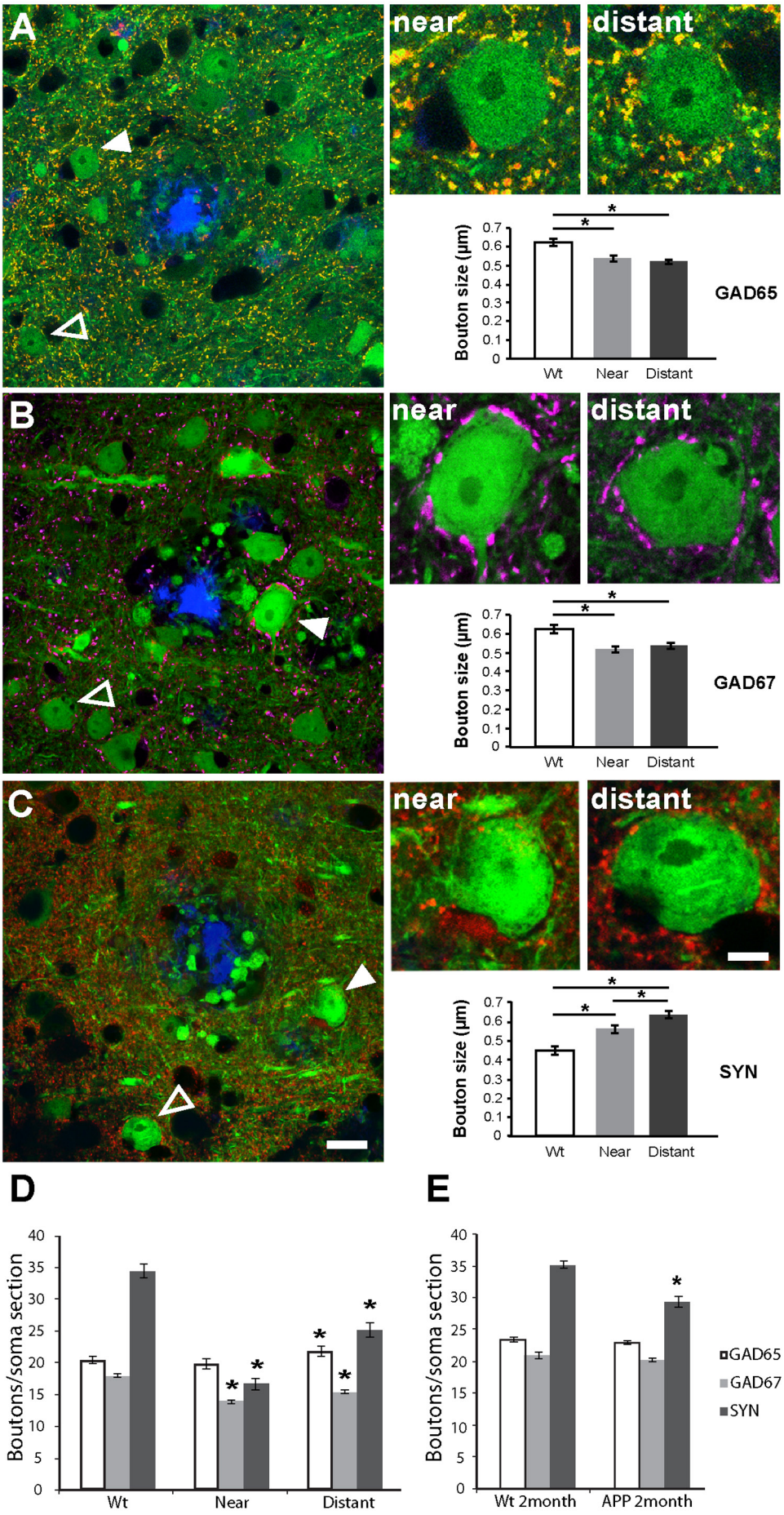

A
Pre
VGlut-1bouton size (µm²)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
***
*
Control
Plaque-free
Periphery
Plaque
B
Pre
VGAT bouton size (µm²)
**
***
C
AD
***
D
AD
***
***
E
Tg
*
*
F
Tg
***

Figure 4.6

Representative sections labeled with GAD65 (A), GAD67 (B) and SYN (C) show perisomatic bouton staining in soma that are near Aβ plaques (filled arrowheads) or more distant (clear arrowheads). Higher magnification of indicated soma that are near (middle column) or distant (right column) show the distribution of perisomatic boutons. There was a significiant reduction in bouton size from Wt values in both GAD65 and GAD67 labelled sections ($p < 0.05$). Conversely, SYN bouton size significantly increased compared to Wt values ($p < 0.05$). (D) There are significantly fewer perisomatic GAD67 and SYN boutons on soma that are near, as well as soma that are distant to Aβ plaques compared to wildtype values ($p < 0.01$). There was no change in GAD65 perisomatic bouton number. (E) In 2 month old animals, there were also significantly fewer SYN boutons per soma ($p < 0.05$). Error bars denote standard error of the mean. Scale bar = 20μm (left), 5μm (right).


A
near
distant
Bouton size (µm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Wt
Near
Distant
GAD65
B
near
distant
GAD67
C
near
distant
SYN
D
Boutons/soma section
40
35
30
25
20
15
10
5
0
Wt
Near
Distant
E
Wt 2month
APP 2month
GAD65
GAD67
SYN

noteworthy that even at two months of age, there were significantly fewer (29.6 ± 1.2; $p < 0.05$; Figure 4.6 E) SYN-ir boutons/soma in APP/PS1 +/– mice (n = 113 somata) compared to age-matched littermates (35.1 ± 0.7, n = 86 somata), but no significant change in GAD67- or GAD65-ir bouton numbers (Figure 4.6 E).

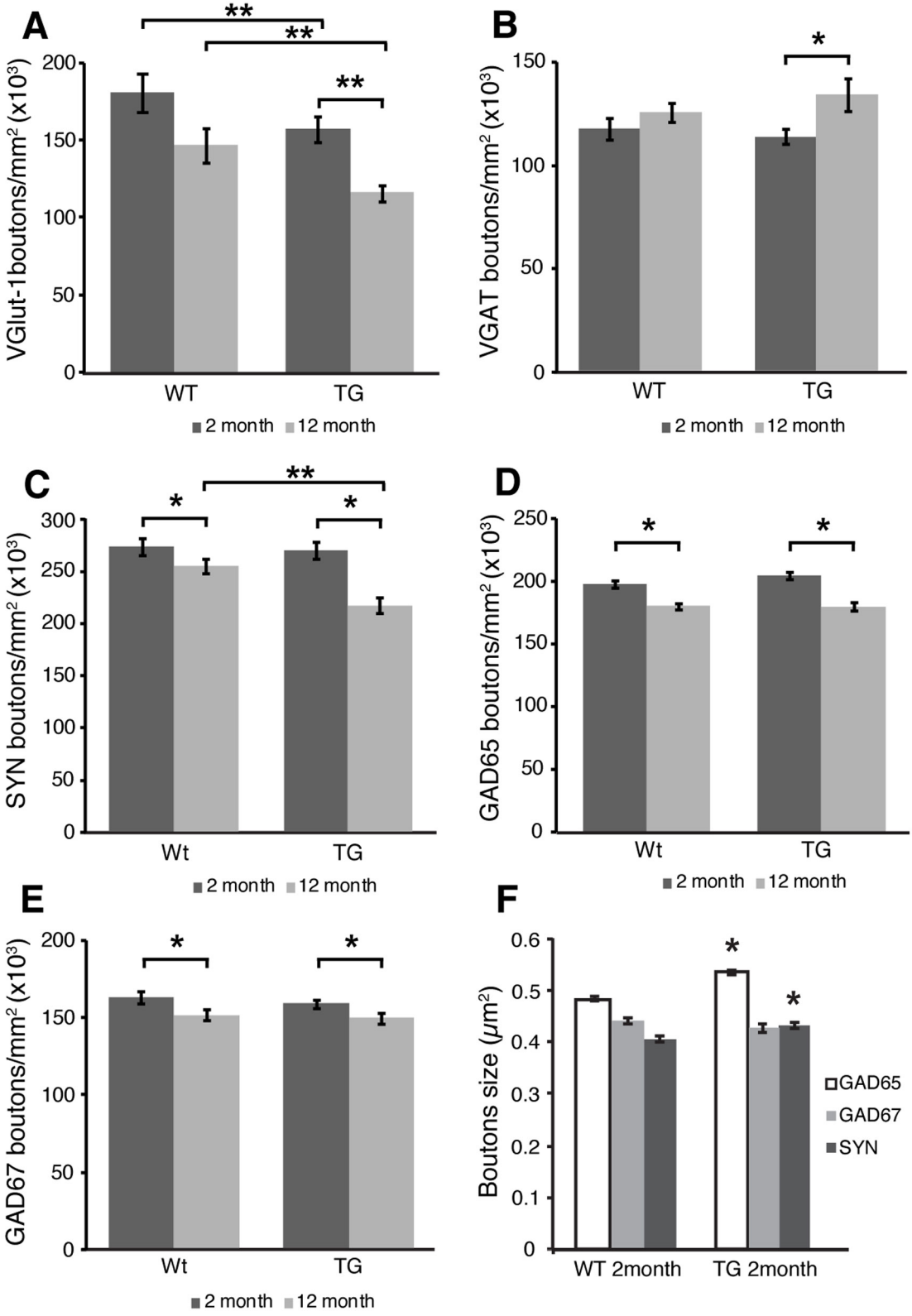
In 12 month-old APP +/– mice, SYN-ir perisomatic boutons synapsing with both near (0.566μm^2) and distant (0.641μm^2) somata were significantly larger than controls (0.449μm^2, $p < 0.01$; Figure 4.6 C), but GAD65- and GAD67-ir boutons on near (0.543μm^2, 0.521μm^2) and distant (0.564μm^2, 0.538μm^2) somata were significantly smaller than controls (0.648μm^2, 0.624μm^2 for GAD65 and GAD67 respectively, $p < 0.01$; Figure 4.6 A, B). Furthermore, there was no significant difference in the bouton size of perisomatic GAD67-ir ($p = 0.13$) and GAD65-ir ($p = 0.22$) between near and distant somata, whereas SYN-ir boutons on distant somata were significantly larger than those on near somata ($p < 0.01$).

Excitatory presynaptic sites are significantly reduced before Aβ deposition begins

When pre-plaque two month-old transgenic mice were compared to control littermates, there was no significant difference in cortical presynaptic marker density except for VGlut1 boutons ($p < 0.01$; Figure 4.7 A). In all APP/PS1 animals, presynaptic bouton density in plaque-free neuropil decreased with age, however, the densities of VGlut1 and VGAT in control cortex from 12 month-old animals were not significantly different from their two month-old counterparts ($p < 0.05$; Figure 4.7 A-E). In two month-old APP +/– animals, the mean sizes of SYN-ir (0.432μm^2) and GAD65-ir (0.537μm^2) boutons were significantly larger than controls (0.405μm^2

Figure 4.7

In pre-plaque forming 2 month-old TG animals, there was a significant decrease in total VGlut-1 bouton number from control values (A). Moreover, there was a significant age-related loss in SYN (C), GAD65 (D) and GAD67 (E) bouton numbers, however this was restricted to just TG animals for VGlut-1 boutons (A), while 12 month-old TG animals had more VGAT-ir boutons than littermate controls (B). When perisomatic bouton size in 2 month-old animals was analyzed, there was a significant increase in both GAD65 and SYN bouton size (*$p < 0.01$; **$p < 0.001$). Error bars denote standard error of the mean.


A
VGlut-1boutons/mm² (x10³)
200
150
100
50
0
**
**
**
WT
TG
2 month
12 month
B
VGAT boutons/mm² (x10³)
150
100
50
0
*
WT
TG
2 month
12 month
C
SYN boutons/mm² (x10³)
300
250
200
150
100
50
0
*
**
*
Wt
TG
2 month
12 month
D
GAD65 boutons/mm² (x10³)
250
200
150
100
50
0
*
*
Wt
TG
2 month
12 month
E
GAD67 boutons/mm² (x10³)
200
150
100
50
0
*
*
Wt
TG
2 month
12 month
F
Boutons size (μm²)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
WT 2month
TG 2month
GAD65
GAD67
SYN

and 0.483μm^2, respectively; $p < 0.01$), whereas there was no significant change in GAD67-ir mean bouton size ($p = 0.12$; Figure 4.7 F).

4.4 Discussion

In the present study, a relative sparing of VGAT-ir GABAergic presynaptic sites in human preclinical and end-stage AD cases, as well as in APP/PS1 mice was demonstrated. Conversely, there was a general decrease in VGlut-1-ir excitatory presynaptic boutons in end-stage AD that was less pronounced in preclinical AD cases and transgenic mice, consistent with a progressive loss of synapses as disease advances (Figure 4.2; Terry et al. 1991; Scheff et al. 2006). Supporting this finding, this study also showed a decrease in VGlut-1 and synaptophysin, but not VGAT protein levels in aged TG mice compared to controls. Similarly, despite an overall decline in presynaptic sites, as determined by decreased synaptophysin immunoreactivity in APP/PS1 +/– YFP +/– mice, GAD65-ir and GAD67-ir bouton numbers remained unchanged in plaque-associated neuropil. When the relative size of VGlut-1-ir puncta in preclinical AD cases and transgenic mice was compared to that of controls, there was a significant increase restricted to plaque-free neuropil, which was absent in end-stage AD cases. Interestingly, both preclinical and end-stage AD cases had increased VGAT bouton size in plaque-free and plaque-rich neuropil.

It has previously been shown that various human APP mutant TG mouse models exhibit neuronal pathology which more closely resembles the initial stages of AD than end-stage disease, and are thus likely to be of greatest validity in modelling early disease processes and alterations (Woodhouse et al. 2009ba; Vickers et al. 2009). Consistent with this, the present study shows that in TG mice, there was actually an increase in the total number of excitatory presynaptic terminals in Aβ plaque-free neuropil compared to controls, whereas there were significant decreases in plaque-rich areas in the same mice. Likewise, preclinical AD cases, unlike end-

stage AD, showed no excitatory bouton reductions in plaque-free neuropil. Previous studies have reported increased glutamatergic presynaptic bouton numbers (Scheff and Price 2003; Bell et al. 2007) as well as excitatory synapse machinery- and plasticity-related genes and proteins (Counts et al. 2006; Williams et al. 2009) in patients at early stages of AD. Such compensatory enhancements, or remodelling, of excitatory signalling during incipient AD are consistent with an early and pronounced loss of glutamatergic synapses during AD progression (Terry et al. 1991; Bell et al. 2006).

Similar to this study's findings of reduced VGlut-1 density in end-stage AD plaque-rich neuropil, decreases in cortical VGlut-1 immunoreactivity and protein levels have previously been shown to correlate well with cognitive deficits in end-stage human AD (Kashani et al. 2008; Chen et al. 2011a). Likewise, studies in TG mice also show reduced glutamatergic presynaptic boutons and VGlut-1 protein levels in Aβ plaque-rich regions (Bell et al. 2003, 2006; Cassano et al. 2012). Furthermore, glutamate concentrations in human AD tissue (Rupsingh et al. 2011) and TG mice (Cassano et al 2012) were reduced while glutamate transporters were down-regulated (Chen et al. 2011a), further supporting the selective loss of glutamatergic signalling as a pathological process in AD. In contrast, this study found no significant reductions in inhibitory presynaptic sites, even in end-stage AD cases, in accord with previous studies of AD and TG tissue (Davies 1998; Bell et al. 2003, 2006). One potential explanation for this disparity is that Aβ accumulations have been observed in VGlut-1 positive synaptosomes isolated from human cortex, but not inhibitory VGAT synaptosomes (Sokolow et al. 2012). This is consistent with the previous finding that Aβ does not bind inhibitory postsynaptic sites (Lacor et al. 2007), and may potentially contribute to neurotransmitter-selective synapse loss observed in AD

(Bell et al. 2006). Alternatively, the selective damage to the glutamatergic system may be related to the preferential degeneration of cortical pyramidal neurons in this condition (Morrison and Hof, 2007).

Concomitantly, a general increase in the size of excitatory puncta in plaque-free neuropil in preclinical AD cases and TG mice, which was absent in end-stage AD, was found in this study. Interestingly, both preclinical and end-stage AD cases showed increases in inhibitory bouton size in plaque-free and plaque-rich neuropil. Although light microscopy lacks the resolving power to distinguish individual presynaptic sites which, if clustered, may appear as a single punctum (Braitenberg and Schüz, 1998, p. 34), previous studies have used this approach to approximate bouton size in brain sections (Marty et al. 2004; Chattopadhyaya et al. 2004, 2007). *In vitro* studies applying Aβ oligomer to cultured neurons have found size increases in synaptophysin-positive presynaptic terminals within six hours of treatment, preceding any overt decreases in overall numbers (Klein et al. 2007). Larger bouton sizes may imply bigger presynaptic vesicle pools (Wilson et al. 2005) with a higher release probability (Branco et al. 2010), and thus a stronger inhibitory/excitatory post-synaptic potential. In the context of the present findings in AD and TG brains, it remains unclear whether such dynamic changes in the size/number of boutons represent part of a secondary compensation for failing excitatory signalling, or a primary driver of pathological cognitive decline. The balance of excitatory to inhibitory network activity is crucial for normal cognitive function (Isaacson and Scanziani, 2011), suggesting that selective perturbations in excitatory glutamatergic signalling in early AD (Bell et al. 2006; Rissman and Mobley 2011) would need to be closely compensated by alterations in inhibitory signalling (Palop et al. 2007).

Given the chronic course of AD, however, initially beneficial responses may eventually drive abnormal activity, resulting in an increase in the number of 'hyperactive' or 'silent' neurons in Aβ plaque-rich tissue (Busche et al. 2008), which could drive epileptogenic activity (Palop et al. 2007; Larner et al. 2010). One possibility is that following a decline in brain activity that is associated with normal ageing, there is a homeostatic disinhibition due to downregulation of GABA-related genes (Gleichmann et al. 2012). Interestingly, when young and old control animals were compared, there was an age-related reduction in both GAD65-ir and GAD67-ir bouton densities. This compensatory response could be even more pronounced in early AD (Tan et al. 2010), where age and Aβ would act in concert to dampen NMDA-dependent synaptic activity (Shankar et al. 2007; Gengler et al. 2010). Paradoxically, this disinhibition would then predispose some neurons to becoming hyperexcitable due to Aβ-mediated calcium dysregulation and eventually result in aberrant network dynamics (Busche et al. 2008).

Therefore, perisomatic inhibitory input on neuronal somata adjacent to, or distant from Aβ deposits was analysed, to determine if distance from plaque affected individual somatic innervation. Previous reports have shown significant decreases in dendritic spine numbers (Spires-Jones et al. 2007) and synaptophysin immunoreactivity (Dong et al. 2007) distant to Aβ plaques. In this study, reductions in GAD67-ir and GAD65-ir bouton density were restricted to within Aβ plaques, with no decreases in the plaque-associated periphery or plaque-free neuropil, similar to previous work in AD and APP/PS1 tissue (Garcia-Marin et al. 2009). Although there were slightly fewer GAD67-ir boutons on both 'near' and 'distant' somata than in controls, no significant change in the number of GAD65-ir boutons was found (Figure 4.6). As GAD65 is almost exclusively localized to presynaptic terminals

(Kanaani et al. 2002), this could signify that while overall cortical GABAergic innervation is intact in old APP/PS1 +/– YFP +/– mice, the ratio of GAD67-ir to GAD65-ir perisomatic terminals could be changed.

While total presynaptic marker density in young APP/PS1 +/– Thy1-YFP +/– mice was also normal, there were reductions in the number of perisomatic SYN-ir but not GAD65-ir or GAD67-ir boutons. The average size of SYN-ir presynaptic boutons was also significantly increased, perhaps as a compensatory response. Indeed, when comparing young and old control animals, an age-related increase in GABAergic bouton size and decrease in bouton density was observed. In contrast, SYN-ir bouton size on both 'near' and 'distant' somata in old APP/PS1 +/– Thy1-YFP +/– mice was significantly larger than controls, whereas GAD65-ir and GAD67-ir boutons were significantly smaller in both soma groups. This enlargement corresponds well with ultrastructural data from early to moderate stages of AD (Scheff and Price 2003) and may represent a compensatory response to preserve declining synaptic function. Similarly, the smaller GABAergic bouton sizes that were observed in plaque-associated neuropil may indicate a homeostatic reduction in presynaptic GABA concentration or vesicle pool size in response to decreased network activity (Hartman et al. 2006) due to loss of spines or presynaptic sites. Although such plasticity may play a neuroprotective role initially, given the chronic progressive nature of AD, it may ultimately contribute to signalling imbalances and network abnormalities (Palop et al. 2007).

In conclusion, this study has shown a selective preservation of inhibitory presynaptic bouton density except inside Aβ plaques, in both human AD cases and transgenic mouse models. Concomitantly, there were significant reductions in both excitatory

(VGlut1-ir) and total (SYN-ir) bouton density extending to plaque-free neuropil as well. Furthermore, this study showed potentially compensatory changes in synaptic morphology in Aβ plaque-rich neuropil, namely a reduction in both GAD65-ir and GAD67-ir bouton sizes coupled with an increase in SYN-ir bouton size in transgenic animals. Although such adaptive structural plasticity may play a neuroprotective role in normal aging, in AD it may ultimately contribute to signalling imbalances and network abnormalities (Palop et al. 2007). Therefore, novel drug treatments aimed at modulating GABA receptor activity, or glutamatergic-GABAergic signalling imbalances, could prove fruitful in addressing network dysfunction in AD.

5. Increased GAD activity in aged APP/PS1 animals is due to increased astrocyte GABA production

5.1 Introduction

In the CNS, the rate-limiting step in GABA production is catalysed by the two isoforms of glutamate decarboxylase (GAD): the 65kD form that is enriched in synaptic boutons, and the constitutively active 67kD isoform which is found in both terminals and cell somata (Soghomonian and Martin, 1998; Kanaani et al. 2002, 2010). GAD67 is responsible for over 90% of cortical GABA production (Asada et al. 1997) and is transcriptionally regulated in an activity-dependent manner: for example, blocking activity by administering tetrodotoxin can severely decrease tissue GAD levels (Chattophadyaya et al. 2007; Lau and Murthy 2012). Furthermore, GAD can also be influenced by global GABA concentrations, with higher GABA downregulating GAD protein levels (Soghomonian and Martin 1998). Conversely, GAD65 accounts for only about 10% of baseline GABA activity and is mostly localized to GABAergic boutons where it may be recruited during periods of heightened GABAergic transmission. In development, neuronal activity is crucial for inhibitory synapse maturation and proper inhibitory axonal morphology, as has been shown in a variety of activity blockade paradigms in dissociated hippocampal cultures (Hartman et al. 2006), organotypic slices (Marty et al. 2004), as well as *in vivo* (Chattophadyaya et al. 2007).

Apart from the total number of presynaptic terminal sites available, another factor mediating signalling strength and efficacy is the available pool of vesicles and their content of neurotransmitter (Hartman et al. 2006). Interestingly, Aβ has recently been shown to disrupt synaptophysin binding to synaptobrevin (Russel et al. 2012), a

crucial regulator of excitatory vesicle exocytosis and endocytosis (Kwon and Chapman 2010). In the previous chapter, TG mice exhibited a pronounced loss in synaptophysin puncta and protein levels along with decreases in VGlut-1 transporter levels, which together imply reduced excitatory vesicle filling and depletion of the available vesicle pool for exocytosis (Wilson et al. 2005; see Chapter 4). In the case of GABAergic synapses, the key determinant of vesicular filling, and thus synaptic strength, is the amount of GAD67 available (Lau and Murthy 2012).

Post-mortem studies in human AD brain have reported unaltered levels of GAD enzyme activity (Reinikainen et al. 1988) and GAD67 mRNA levels (Boissiere 1998) compared to control cases. The main GABA transporter (GAT-1) was also found to be unaffected in AD tissue compared to controls (Nagga et al. 1999). Although some reports found only slight changes in cortical GABA levels in end-stage AD patients (Lowe et al. 1988), more recent work has shown significant GABA reductions in areas particularly affected by AD such as temporal cortex (Siedl et al. 2001). Given the importance of inhibition to maintain cortical function and coordinate network activity (Isaacson and Scanziani, 2011), it is clearly important to determine the vulnerability of the GABAergic system to AD pathology. Therefore, it was the aim of this study to determine if the amount of GAD enzyme and its specific activity differed significantly in Aβ plaque-rich neuropil in TG mice from control tissue.

5.2 Materials and Methods

Synaptosome preparation

Crude synaptosomes were obtained by a modified version of Dunkley and colleagues' method (2008). Briefly, whole APP/PS1 and wildtype brains were harvested at two, six and twelve months (*n*=6 each, for each time point) and the cortex and cerebellum were dissected. The tissue was hand-homogenized in 10vol (wt/vol) of sucrose buffer (0.32M sucrose, 1mM EDTA, 5mM Tris, 0.25mM Dithiothreitol, ph 7.4) supplemented with a protease inhibitor cocktail (Roche, Germany) at 0°C in a glass Teflon homogenizer with 12-15 strokes and then centrifuged at 1000*g* for 15 min at 4°C (SS-34 rotor in Sorvall RC5C Plus). The pellet (P1) was frozen and stored while the supernatant (S1) was then resuspended in fresh sucrose buffer and centrifuged for a further 30mins at 15000*g* at 4°C. The resulting pellet (P2) was then resuspended in either freshly made Krebs Ringer phosphate (KRP) buffer (see Appendix A) for enzyme assays, or in fresh sucrose buffer at 0°C and supplemented with a protease inhibitor cocktail. This pellet contains the bulk of synaptosomes as well as some myelin and mitochondria (Dunkley et al. 2008). It also contains contaminating re-sealed plasma membranes from glial origin ('gliasomes') that are synaptically active (Stigliani et al. 2006).

Crude synaptosomes were further purified by resuspending P2 and layering it onto a discontinuous Percoll gradient comprising layers of 3%, 10%, 15% and 23% (vol/vol) Percoll (GE Healthcare; see Dunkley et al. 2008). The gradient was then centrifuged for 8 min at 31000*g* at 4°C in a Sorvall WX Ultra90 (70.I TI rotor) and the resulting fractions were then carefully collected. The fraction collected at the

interface of the 3% and 10% layers (F2) has been characterized as predominantly glial membranes, while those between 10% and 15% (F3) and 15% and 23% (F4) were pooled as purified synaptosomes (Stigliani et al. 2006; Dunkley et al. 2008). Samples used for enzymatic activity assays were used fresh immediately. Aliquots were also frozen in liquid nitrogen for further protein analysis.

Immunoblotting

For Western blot analysis of synaptic proteins, crude synaptosomes from 2 and 12 month-old APP/PS1 animals and wildtype controls (n = 5 each, 3 repeats per sample) were harvested as outlined above. The protein concentration of samples was determined using the Qubit fluorescent protein assay kit (Invitrogen) in triplicate. Samples were then separated by SDS-PAGE (20μg protein/lane; 12% NuPAGE Novex Bis-Tris gels; Invitrogen). Protein gels were transferred to a PVDF membrane (Bio-Rad Laboratories), and membranes were blocked for 2 hours in 5% commercial skim milk powder. One gel from each experiment was stained with Coomassie blue (Brilliant Blue R, Sigma-Aldrich) to compare replicates for consistency. Membranes were incubated overnight in primary antibodies, including combinations of anti-GAD67 (1:2500, Millipore), anti-GAD65 (1:2500, Millipore), anti-synaptophysin (1:5000, Millipore) and anti-β-tubulin (1:5000, Sigma-Aldrich). Membranes were washed in Tris-buffered saline with 0.1% Tween-20 (Sigma-Aldrich). Species-appropriate horseradish peroxidase conjugated secondary antibodies (1:2000, DAKO) were applied and visualized with a chemiluminescent peroxidase substrate kit (Millipore).

GAD enzymatic activity assay

GAD activity was assessed using a modified version of Ross and Craig's protocol (1981). Briefly, after the initial separation of crude synaptosomes, P2 was resuspended in freshly made KRP buffer and centrifuged for 10 min at 48000*g* at 4°C in a Sorvall WX Ultra90 (70.I TI rotor). The supernatant (S3) was discarded while the pellet (P3) was resuspended in fresh KRP buffer. This process was repeated and the final P4 pellet was reconstituted in KRP buffer, 100µl of which was used in the GAD assay. Each assay tube contained 800µl of KRP buffer, 100µl of synaptosome sample, and 100µl of cofactor and substrate mix (0.5mM pyridoxol-5-phosphate, Sigma-Aldrich; 0.5mM reduced glutathione, Sigma-Aldrich; 20mM L-glutamic acid, Invitrogen; 4µM L-[^{14}C-(U)]-glutamic acid, specific activity 0.1µCi/mol, Perkin Elmer). All tubes were sealed by a rubber stopper with a centre well (Kimble Chase Kontes) in which filter paper (Whatman 934-AH) pre-soaked with 30µl of 1M benzethonium hydroxide (Sigma-Aldrich) was placed. The enzyme reaction was allowed to continue for 60 min at 37°C in a shaking water bath. Termination of enzyme activity was accomplished by injecting 50µl of 3M sulphuric acid through the rubber stopper, without permitting the acid to touch the filter paper. The acid inhibits GAD activity and liberates $^{14}CO_2$ which is trapped by the pre-soaked filter paper. After an equilibration period of 60mins, the rubber sleeve was removed and the filter paper was quickly placed in scintillation vial to which 5ml of scintillation cocktail (Amersham Biosciences) was added before scintillation was measured in a Perkin Elmer Tri-Carb 2800TR liquid scintillation counter.

5.3 Results

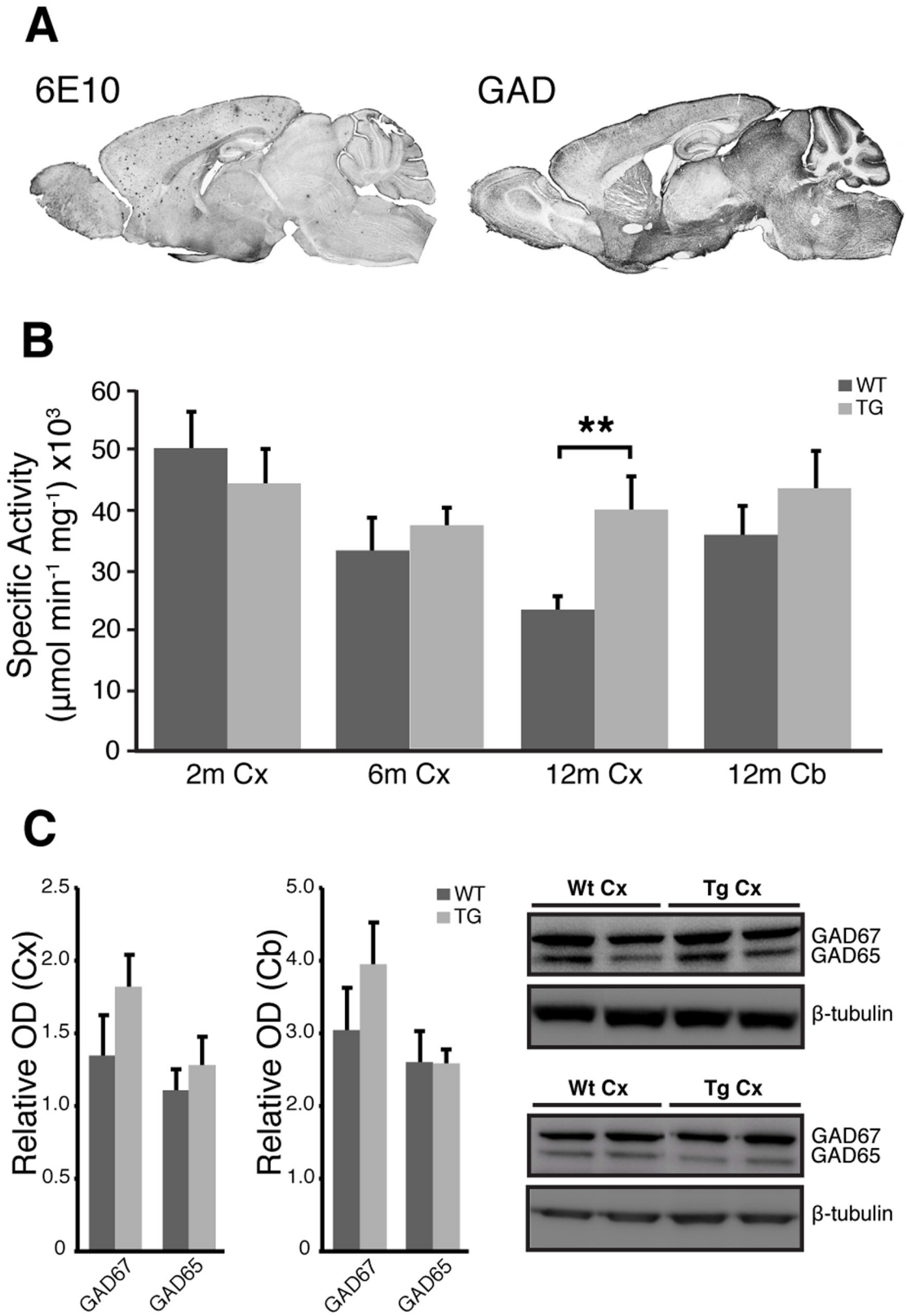
Increase in GAD specific activity in Aβ plaque-rich neuropil of TG mice

In TG mice, Aβ deposits have been reported to be mainly restricted to the cortex, hippocampus and parts of the olfactory bulb, whereas other brain regions such as the cerebellum are plaque-free (Borchelt et al. 1997; Garcia-Alloza et al. 2006a; Figure 5.1 A). Therefore, this study compared cortical to cerebellar tissue to determine whether the presence of fibrillar Aβ had a region-specific effect on the enzymatic activity of GAD, which is ubiquitously expressed in both cortex and cerebellum (Soghomonian and Martin 1998; Figure 5.1 A). Crude synaptosome tissue from both the cortex and cerebellum of two, six and twelve month-old TG and littermate control animals was extracted and prepared for enzyme assays as previously described. There was almost a doubling of GAD specific activity in 12 month-old TG cortex compared to age-matched wildtypes (39.4 ± 5.6 versus $22.8 \pm 2.4 \times 10^3$ $\mu mol.min^{-1}.mg^{-1}$ for TG and wildtype, respectively, Figure 5.1 B; $p < 0.01$), whereas there was no significant difference in cerebellar tissue taken from the same animals. When GAD activity in younger pre-plaque TG cortex (2 month-old) or TG cortex with relatively few plaques (6 month-old) was examined, there were no significant differences compared to age-matched wildtype cortical tissue (Figure 5.1 B).

An aliquot of each crude synaptosome preparation was also taken for immunoblotting to determine if changes in GAD protein levels could account for differences in activity. Two distinct bands for GAD65 and GAD67 labelling were present at 65 and 67 kDa, respectively, and β-tubulin (55 kDa) was used as a loading control. There were no significant changes in GAD protein levels in either 12 month-old cortex (Cx) or cerebellum (Cb) between TG and wildtype animals (Figure 5.1 C), or in 2 and 6 month-old animals (data not shown).

Figure 5.1

(A) Sagittal 12 month-old TG sections DAB-stained for Aβ deposits (6E10) or for the GABA synthesizing enzyme GAD showing the uniform distribution of GAD throughout the cortex and dense staining within the molecular layer of the cerebellum, while Aβ deposits were almost exclusively found in the cortex and olfactory bulb. (B) The specific activity of GAD was significantly increased in 12 month-old TG cortical synaptosomes (Cx) but not in cerebellum (Cb) or in younger TG cortex (**$p < 0.01$). (C) This difference in the specific activity of GAD could not be explained by any differences in overall GAD65 or GAD67 protein levels as determined by immunoblotting.


A
6E10
GAD
B
Specific Activity
(µmol min-1 mg-1) x10³
60
50
40
30
20
10
0
WT
TG
**
2m Cx
6m Cx
12m Cx
12m Cb
C
Relative OD (Cx)
2.5
2.0
1.5
1.0
0.5
0
GAD67
GAD65
Relative OD (Cb)
5.0
4.0
3.0
2.0
1.0
0
WT
TG
Wt Cx
Tg Cx
GAD67
GAD65
β-tubulin
Wt Cx
Tg Cx
GAD67
GAD65
β-tubulin

Increased GAD specific activity in Aβ plaque-rich neuropil is predominantly of glial origin in TG mice

Next, this study examined if the increased GAD specific activity in TG cortex synaptosomes was due to a redistribution of cytoplasmic GAD into synaptic terminals (Kanaani et al. 2010) in TG cortex as there were no overall changes in cortical GAD protein levels. Therefore, the enzyme assay was performed using the P1 fraction, which contains most cytoplasmic proteins as well as non-soluble proteins, nucleic acids and cell debris (Dunkley et al. 2008). There was no significant difference in either GAD specific activity (Figure 5.2 A) or GAD protein levels (Figure 5.2 B) between TG and littermate controls in the P1 fraction.

Therefore, the 12 month-old crude synaptosome extracts were further purified in order to separate any contaminating glial membranes ('gliasomes'; Stigliani et al. 2006). The two resultant fractions (F2: glial membranes; F3+4: pure synaptosomes, Figure 5.3 A) were then used for the GAD enzyme assay. Interestingly, there was no difference in GAD specific activity between TG and wildtype pure synaptosomes ($p = 0.64$). Conversely, TG glial GAD specific activity was double that of wildtype (229.9 ± 29.6 versus $115.5 \pm 20.9 \times 10^3$ $\mu mol.min^{-1}.mg^{-1}$, respectively. Figure 5.3 B; $p < 0.01$). When immunoblots of purified glial and synaptosomal fractions were compared, there was no significant difference in GAD65 or GAD67 protein levels (Figure 5.3 C, D).

Figure 5.2

(A) There was no difference in the GAD specific activity of the 12 month-old TG P1 fraction compared to wildtype levels. (B) Furthermore, there was no significant difference in GAD67 protein levels between TG and control cortical P1 fraction.

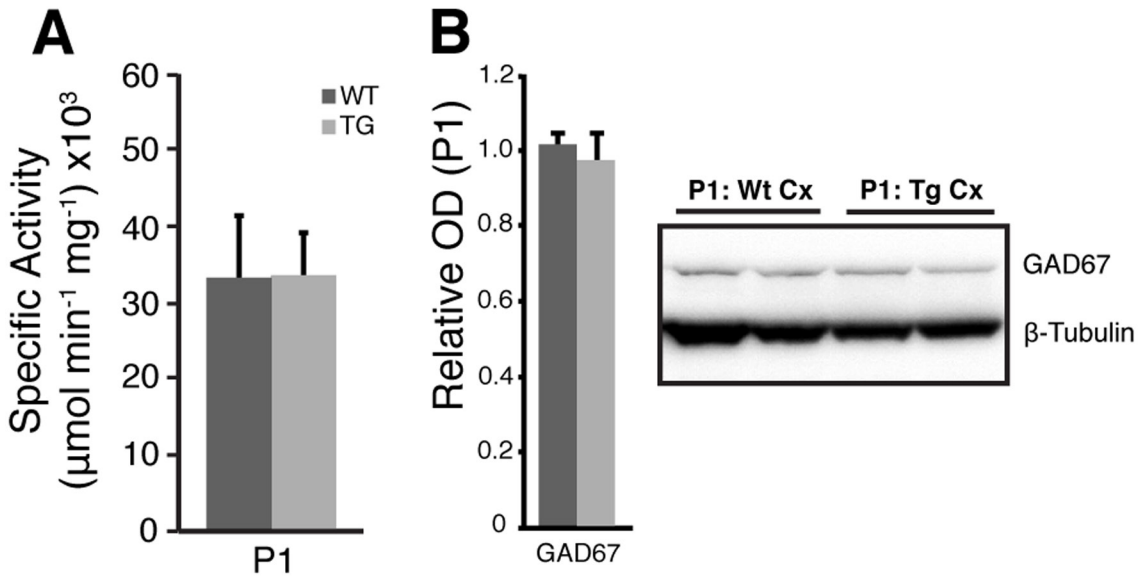
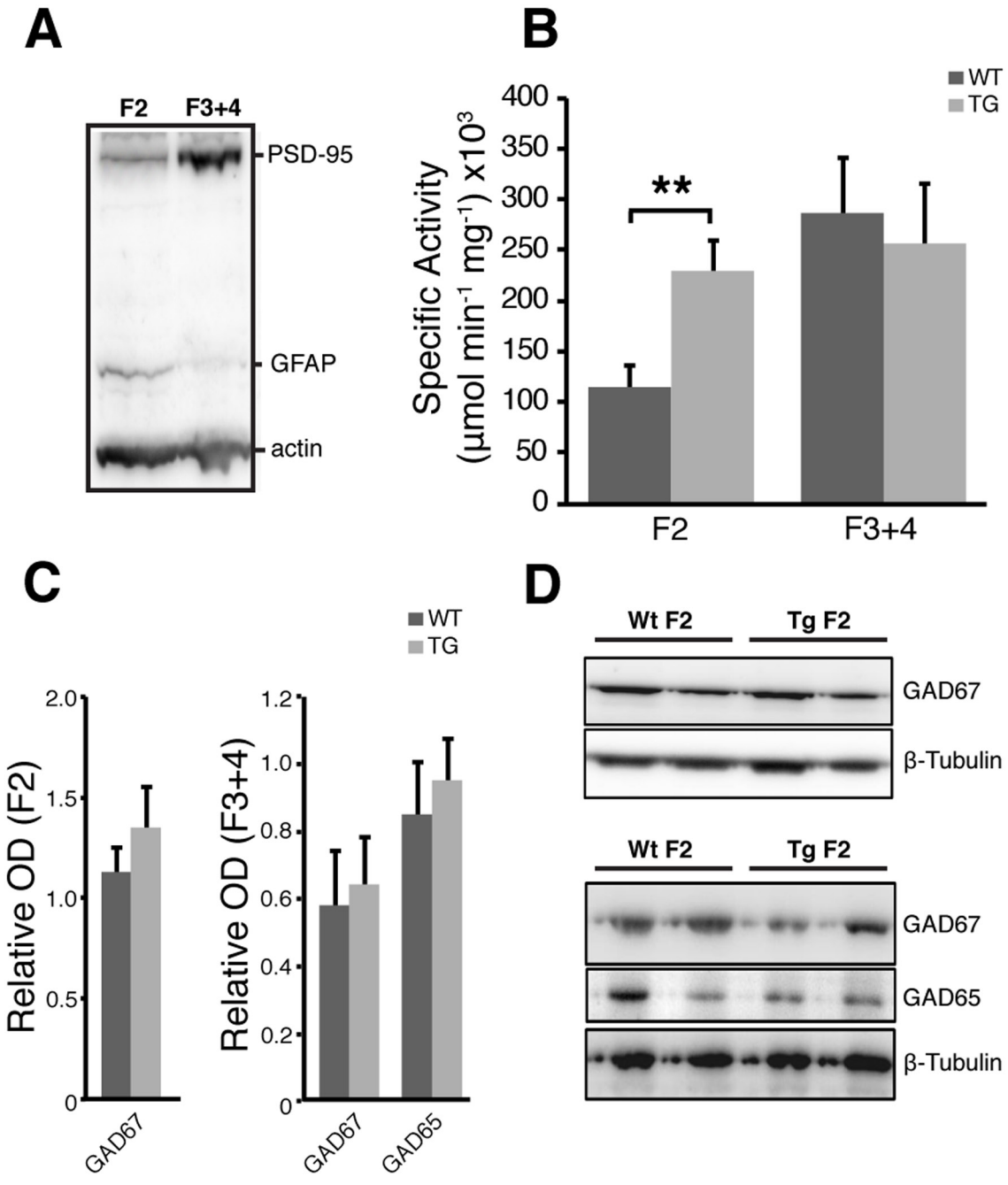

A
Specific Activity
(µmol min-1 mg-1) x10³
60
50
40
30
20
10
0
WT
TG
P1
B
Relative OD (P1)
1.2
1.0
0.8
0.6
0.4
0.2
0
GAD67
P1: Wt Cx
P1: Tg Cx
GAD67
β-Tubulin

Figure 5.3

(A) A sample immunoblot from the glial enriched fraction (F2) and the synaptosome enriched fractions (F3+4) obtained from discontinuous Percoll gradients of 12 month-old TG crude synaptosomes, showing purified synaptosomes contain no glial contamination as evidenced by no GFAP labelling. (B) The TG glial fraction had significantly higher GAD activity compared to WT littermate controls whereas there was no difference in the enriched synaptosomes fraction (**$p < 0.01$). There was no significant difference in GAD protein levels compared to wildtype tissue as determined by immunoblotting (C, D).


A
F2
F3+4
PSD-95
GFAP
actin
B
WT
TG
Specific Activity
(µmol min-1 mg-1) x10³
400
350
300
250
200
150
100
50
0
**
F2
F3+4
C
WT
TG
Relative OD (F2)
2.0
1.5
1.0
0.5
0
GAD67
Relative OD (F3+4)
1.2
1.0
0.8
0.6
0.4
0.2
0
GAD67
GAD65
D
Wt F2
Tg F2
GAD67
β-Tubulin
Wt F2
Tg F2
GAD67
GAD65
β-Tubulin

5.4 Discussion

This study compared the specific activity of glutamate decarboxylase in synaptosomes isolated from plaque-rich and plaque-free brain regions in TG mice relative to wildtype littermates. There was a significant doubling in GAD activity in twelve-month old TG cortex compared to wildtype animals, with no significant differences between the two in plaque-free regions or in younger animals. Furthermore, this study shows that the glial fraction in synaptosomes (Stigliani et al. 2006) was the source of this increased GAD activity. There were no changes in total GAD protein levels in cortical synaptosomes compared to wildtype controls, consistent with the previous finding of no reductions in inhibitory presynaptic sites or the density of GAD65-ir and GAD67-ir boutons in TG cortex (see Chapter 4).

Therefore, this study aimed to determine if GAD activity in TG synaptosome samples was different from wildtype controls. Surprisingly, a significant increase in GAD activity was detected in twelve month-old TG animals compared to controls. This was restricted to the Aβ plaque-laden cortex and not observed in the cerebellum, which normally lacks Aβ deposition in TG animals. Furthermore, when younger animals with limited (6 months) or no (2 months) Aβ deposits were analyzed, there was no difference in GAD activity, suggesting that increased GAD activity occurs in parallel with age-dependent accumulation of fibrillar Aβ. Because the increase in GAD activity was restricted to cortical synaptosomes, they were further purified using discontinuous Percoll gradients, which yielded two main fractions: glial-derived membranes (gliasomes) and pure (neuronal) synaptosomes (Dunkley et al. 2008). Further analysis revealed that GAD activity was doubled in the gliasome fraction, whereas there was no significant difference in the neuronal synaptosomes, strongly implying that the overall increase in GAD activity in TG cortex was due to a

specific increase in glial GAD activity. However, the GFAP-positive glial fraction had similar GAD67 protein levels to that of controls.

Additional to their well-documented role in neurotransmitter re-uptake from the synaptic cleft, astrocytes have also been shown to release synaptically active transmitters and peptides, dubbed 'gliotransmission' (Hamilton and Attwell 2010). GABA release by astrocytes has been shown in slice preparations from distinct brain regions such as cerebellum (Lee et al. 2010) and hippocampus (Heja et al. 2012), as well as in human astrocyte cultures (Lee et al. 2011b). Further supporting a role in synaptic signalling, these glial cells express moderately high levels of mRNA for GAD67, GABA transporters 1, 2, and 3 (GAT-1/2/3) as well as the GABA metabolizing enzyme GABA transaminase (GABA-T; Lee et al. 2011a, b).

Although the mechanism of astrocytic GABA release and its physiological function *in vivo* remain hotly debated, it is likely that astrocytes contribute to tonic inhibition in the brain via non-vesicular release of GABA onto post-synaptic neurons (Heja et al. 2012). Interneuron stimulation of $GABA_B$ receptors on astrocytes (Kang et al. 1998; Lee et al. 2010) or direct stimulation by glutamate (Wu et al. 2007; Lee et al. 2011b) has been shown to increase intracellular calcium levels in astrocytes and enhance their release of GABA. This release can be mediated either by a reversal of the GAT transporters such that they release GABA back into the synaptic space (Wu et al. 2007; Lee et al. 2011b; Heja et al. 2012) or by calcium-dependent activation of bestrophin-1 anion channels which drive GABA release (Lee et al. 2010).

Apart from their role in synaptic maintenance and signalling, astrocytes have long been known to participate in reactive gliosis in end-stage AD (Nagele et al. 2004) and numerous transgenic AD models (Rodriguez et al. 2009) where they contribute

to inflammatory toxicity. However, recently it has been suggested that end-stage AD astrogliosis is preceded by astrocyte atrophy and loss of arborization in regions of the brain that AD pathology affects earliest, such as the entorhinal cortex and hippocampus (Yeh et al. 2012). This early astrocytic shrinkage and loss of processes critical for modulating and controlling synaptic transmission could contribute to the synaptic and signalling deficits evident in these brain regions. In this regard, *in vivo* calcium imaging studies in AD transgenic mice have shown early pronounced elevations in astrocyte calcium levels in the hippocampus (Busche et al. 2012) and cortex (Kuchibhotla et al. 2009) in response to increased Aβ levels. These elevated levels were independent of neuronal activity and often resulted in sporadic calcium 'waves' propagated from astrocytes close to Aβ plaques to more distant, electrically-coupled astrocytes (Kuchibhotla et al. 2009). Such hyper-activity in astrocytes could result in the aberrant activation/release of calcium-mediated signalling molecules and transmitters critical for control of synaptic plasticity and function (Hamilton and Attwell 2010).

Since astrocytes are clearly implicated in early and end-stage pathological changes in AD, and in synaptic signalling, there is a clear need for further studies to determine the role of astrocytes in AD signalling dysfunction. The present study suggests that in TG mice, astrocytes may increase their production of GABA in regions with high Aβ load, potentially in compensation for signalling imbalances known to occur in these regions (Palop et al. 2007; Busche et al. 2008). In light of this, it would be interesting to determine what effect the astrogliosis and inflammation that occur in AD have on this potentially neuroprotective role of astroglia and whether similar processes take place in the diseased human cortex.

6. Focal demyelination, oligodendrocyte loss and myelin protein alterations in AD cases and transgenic mouse models

6.1 Introduction

The accumulation of abnormal Aβ into plaques causes a well-characterized cascade of aberrant cytoskeletal changes resulting in the loss of dendritic spines, the formation of dystrophic neurites and, ultimately, neuronal degeneration (Dickson et al. 1999; Tsai et al. 2004; Spires et al. 2005; Woodhouse et al. 2009a, b; Vickers et al. 2009). Recent imaging studies in human AD patients (Salat et al. 2010; Gold et al. 2012; Huang et al. 2012) as well as in transgenic mice (Chen et al. 2011b; Zerbi et al. 2012) have also reported considerable white matter loss and/or abnormalities at early stages of the disease. In addition to gross imaging studies showing white matter hyperintensities indicative of local white matter loss in AD (Bartzokis et al. 2003; Stricker et al. 2009), levels of white matter myelin-associated proteins and cholesterol have also been shown to be reduced in AD and transgenic mice (Svennerholm and Gottfries 1994; Roher et al. 2002; Desai et al. 2009). Although white matter lesions (WML) are a major feature of other inclusion-forming neurodegenerative diseases such as multiple sclerosis (MS) and Charcot-Marie-Tooth disease, demyelination, particularly in the grey matter, has not been extensively studied in AD (Bartzokis 2004; Ihara et al. 2010).

Given myelin's crucial role in the speed and integrity of axonal transmission, it is likely that myelin pathology would significantly contribute to the global cognitive decline characteristic of AD. There are several possible factors that could mediate myelin pathology in the AD: direct toxicity mediated by plaques and/or oligomeric Aβ, as a side effect of axonopathy, or in association with vascular pathology. In the

latter case, beta amyloid, particularly the $A\beta_{1-40}$ peptide, also accumulates and deposits within cerebral vessels as cerebral amyloid angiopathy (CAA; Thal et al. 2008). Although CAA also occurs in a small proportion of the non-demented aged population, it is much more pronounced in AD where it has been shown to correlate with the severity of white matter lesions (Roher et al. 2003; Weller et al. 2009), which in turn, are strongly linked to cognitive performance in AD (Burns et al. 2005). Alternatively, myelin pathology may be the result of direct toxicity of Aβ to oligodendrocytes and/or OPCs (Xu et al. 2001; Chen et al. 2006; Horiuchi et al. 2012) or indirect loss of integral myelin lipids and structural proteins (Svennerholm and Gottfries 1994; Roher et al. 2002; Desai et al. 2009). Finally, local axonopathy due to Aβ plaque-mediated cytoskeletal alteration resulting in dystrophic neurite formation may result in secondary loss of myelin.

Although cortical demyelination in MS cases with concomitant AD has been described (Dal Bianco et al. 2008), few quantitative studies of AD plaque-associated myelin pathology have been reported to date. Therefore, this study aimed to investigate the effect of Aβ plaques on nearby myelin and oligodendrocytes in human cases at various stages of pathological progression and onset ages, as well as plaque-forming transgenic mouse models of AD. Furthermore, the levels of several integral myelin proteins were investigated in purified myelin from APP/PS1 mice at various ages.

6.2 Materials and Methods

Human tissue

Human brain tissue was acquired from the Sun Health Research Institute (Arizona, USA) and the National Health and Medical Research Council Brain Bank (Adelaide, Australia), meeting all necessary ethical approvals as previously described (see Chapter 2). Cases included five presenilin-1 mutation-bearing familial AD cases, eight sporadic AD cases that conform to CERAD criteria for the diagnosis of AD, eight 'preclinical' AD cases and five age-matched control cases lacking Aβ plaques and neurofibrillary pathology (Table 6.1).

Mouse tissue

The brains of twelve month-old Tg2576, APP/PS1 and age-matched wildtype mice (n=5, for each type) were processed as previously described (see Chapter 2). In addition, three and six month-old APP/PS1 and age-matched wildtype mice (n=5 each) were used for the oligodendrocyte lineage analysis.

Immunohistochemistry

Cortical sections were co-immunolabelled with rabbit anti-pan-β-amyloid (Biosource) to visualize Aβ plaques, as previously described (Dickson et al. 1999; Dickson and Vickers 2001), and a fluorescent myelin stain (FluoroMyelin™ Green, Invitrogen). Briefly, Tissue sections were incubated with Fluoromyelin green (1:300; Molecular Probes, Eugene, OR) for 20 minutes at RT in darkness, and washed three times with 0.01M PBS. Ten randomly-chosen fibrillar Aβ plaques per section (layer 5, three sections per case/animal, n=30; section size = 250000μm^2) were assessed for their effects on myelination. For qualitative comparisons, diffuse amyloid deposits

and ‘cotton wool’ plaques were characterized as previously described (Dickson and Vickers 2001; Woodhouse et al. 2009). In brief, cotton wool plaques were defined by their size (>100μm in diameter), clear margins and relative lack of neuritic infiltrate, whereas diffuse plaques were defined as irregularly-shaped deposits of loosely-arranged fibrils without distinct edges.

Mature human oligodendrocyte somata and processes were labelled with rabbit anti-p25α antibody as previously described (Song et al. 2007). Demyelinated nerve tissue was labelled with rabbit anti-degraded myelin basic protein (dMBP) that recognizes epitopes in actively demyelinating regions as previously described (Matsuo et al. 1997). The presence and type of cerebral amyloid angiopathy (CAA) was assessed for all human cases, using the presence of rabbit anti-pan-β-amyloid-labelled Aβ deposits in larger blood vessels (>20μm diameter) and capillaries (<15μm diameter, without rabbit anti-desmin labelled smooth muscle cells) as criteria. Cases that included Aβ deposition in capillaries were considered Type 1 CAA, whereas those with only large blood vessel involvement were considered Type 2 CAA (Thal et al. 2002). Although the CAA staging was not assessed in the samples, a simple grading system based on the mean number of vessels displaying CAA/case was used to categorize severity: severe (>10/section), moderate (5–10/section) or mild (<5/section) (see Table 6.1).

Table 6.1 Human brain cases utilized for immunohistochemistry and analysis.

Type	Age	Gender	Type of CAA	Severity of CAA	Cause of Death
PS1 FAD	44	F	1	+	Pneumonia
PS1 FAD	46	F	1	+	Pneumonia
PS1 FAD	48	M	–	–	Cardiopulmonary arrest
PS1 FAD	49	M	–	–	Cardiopulmonary arrest
PS1 FAD	61	F	–	–	Pulmonary embolus
Sporadic AD	88	M	1	++	AD
Sporadic AD	92	F	1	+	Pneumonia
Sporadic AD	74	F	1	+++	Pneumonia
Sporadic AD	74	M	1	+	Respiratory arrest
Sporadic AD	83	M	1	+	AD
Sporadic AD	66	M	1	++	AD
Sporadic AD	84	F	1	++	AD
Sporadic AD	76	M	1	+++	Cardiac arrest
Preclinical AD	90	M	–	–	Respiratory arrest
Preclinical AD	81	F	–	–	Cardiac arrest
Preclinical AD	84	M	–	–	Cardiopulmonary arrest
Preclinical AD	78	M	–	–	Postoperative
Preclinical AD	91	M	–	–	Cardiac arrest
Preclinical AD	82	M	2	+	Myocardial infarction
Preclinical AD	74	M	–	–	Cardiac arrest
Preclinical AD	74	M	–	–	Cardiac arrest
Control	58	M	–	–	Cardiac arrest
Control	51	M	–	–	Pulmonary embolus
Control	47	M	–	–	Pneumonia
Control	65	M	–	–	Cardiopulmonary arrest
Control	71	M	–	–	Postoperative

+ mild, ++ moderate, +++ severe

Mouse oligodendrocyte precursor cells (OPCs), immature pre-myelinating oligodendrocytes and mature oligodendrocytes were labelled with rabbit anti-PDGRα, rabbit anti-GPR17 and mouse anti-CC1 respectively. Adult-born OPCs in the subventricular zone (SVZ) were labelled with rabbit anti-NG2. All sections were counterlabelled with the pan-oligodendrocyte lineage transcription factor Olig2 and the nuclear label DAPI. A further set of sections was labelled with the axonal marker SMI312 to ascertain if Aβ plaque-associated dystrophic axons were myelinated.

Gallyas silver myelin staining

Both human and mouse sections were also stained with a Gallyas silver staining protocol for myelin as per Pistorio and colleagues (2006) to confirm the fluoromyelin data with a non-flourescent probe for myelination. Briefly, brain sections were immersed in 10% formalin for seven days, followed by a 30 minute wash in 2:1 pyridine and acetic anhydride. Samples were then rehydrated through a 4-step pyridine/water series, followed by two water washes. Brain tissue was then immersed in silver solution for 45 minutes and subjected to three 10 minute washes in 0.5% acetic acid. Sections were placed back in 10% formalin for ~1 hour, following which they were rehydrated by two washes in 0.5% acetic acid. During this step, developer solution was made from solutions A and B (see Appendix 1) and 365µl of 4% PFA per 100ml. Sections were developed for 10-60 minutes (monitoring for staining intensity) and stopped in 1% acetic acid, followed by two additional 10 minute washes in 1% acetic acid; over-stained samples were further differentiated in 0.1% potassium ferricyanide. Sections were then washed in 0.5% sodium thiosulphate for 1 minute followed by three 5 minute washes in distilled water. Sections were then dehydrated through an alcohol/xylene series and mounted on subbed slides with DPX (Sigma-Aldrich, St Louis, MO).

Myelin purification

Myelin was purified from three, six and twelve month-old APP/PS1 and wildtype mouse brains according to Yamaguchi and colleagues' method (2008). Briefly, whole brains were pulverized in a Dounce homogenizer (12-14 strokes) with 10% (wt/vol) of 0.3M sucrose buffer (see Appendix A for composition). The homogenate was centrifuged at 1000*g* for 10 minutes at 4°C (SS-34 rotor in Sorvall RC5C Plus) and the supernatant (S1) was carefully collected. Next, 4mL of S1 was carefully

layered over 4mL of 0.83M sucrose buffer in ultracentrifuge tubes and centrifuged for 90 minutes at 140000*g* (4°C). The layers of crude myelin that formed at the interface of the two sucrose solutions were collected and transferred to a cold Dounce homogenizer. The crude myelin fraction was subjected to osmotic shock by combining with Tris-Cl buffer (5-7 strokes) and centrifuged for 15 minutes at 75000*g* (4°C). The supernatant (S2) was discarded and the pellet (P2) was resuspended in fresh 0.83M sucrose buffer and transferred to ultracentrifuge tubes. After carefully layering fresh 0.3M sucrose buffer over the 0.83M fraction, tubes were centrifuged again for 90 minutes at 140000*g* (4°C). The pure myelin fraction was subjected to a final wash in Tris-Cl buffer and the pellet (P3) was reconstituted in fresh 0.3M sucrose. The protein concentration was determined using the Qubit fluorescent protein assay kit (Invitrogen) in triplicate.

Immunoblots

For Western blot analysis of myelin proteins, pure myelin samples from 3, 6 and 12 month-old APP/PS1 animals and wildtype controls (n = 5 each, 3 repeats per sample) were harvested as outlined above and separated by SDS-PAGE as previously described (see Chapter 2). Briefly, PVDF membranes were probed with combinations of anti-CNPase (1:2500, Abcam), anti-MAG (1:2500, Millipore), anti-MBP (1:5000, Millipore), anti-PLP/DM20 (1:2500, Abcam) and anti-β-tubulin (1:5000, Sigma-Aldrich). Species-appropriate horseradish peroxidase conjugated secondary antibodies (1:2000, DAKO) were applied and visualized with a chemiluminescent peroxidase substrate kit (Millipore).

Imaging and analysis

Images were captured using a Leica DM LB2 fluorescence microscope using a cooled CCD Magnafire (Optronics) camera. To facilitate counting of plaque-associated myelinated fibers, 8-bit greyscale images were inverted using ImageJ software (NIH) (Figure 6.1 C). The numbers of myelinated axons traversing templates corresponding to 50% (plaque core), 100% (plaque edge) and 150% (plaque periphery) of each Aβ plaque's diameter were determined. Myelination in human and mouse controls and in plaque-free neuropil from AD and transgenic cases, defined as being layer 5 but at least 100μm away from the closest Aβ deposit (as per Spires et al. 2005), was also assessed. For these analyses the average plaque size for human AD or transgenic cases was utilized to define a 'pseudo-plaque', that was then randomly placed in images from analogous cortical regions (i.e. layer 5 neocortex). The grid function in ImageJ was used to facilitate non-biased placement of the plaque area template. A subset of images was also acquired using a Zeiss LSM 510 META confocal microscope and deconvolved using Zen image analysis software. The number of myelinated neurites for each zone was normalized to the mean plaque size for each case, to allow proportional comparison between zones. For mouse oligodendrocyte lineage counts, the total numbers of labelled cells in sections of somatosensory (S1) cortex (total area counted = 250000μm^2), underlying corpus callosum (85000μm^2) and SVZ (85000μm^2) was determined (3 sections/animal) and 2D cell density was calculated. Only cells that were counterlabelled for Olig2 and DAPI were counted.

One-way ANOVA with Dunnett's post-hoc test was performed using GraphPad Prism software (version 5.0b), with $p < 0.05$ (CI 95%) considered statistically significant.

6.3 Results

Focal demyelination and oligodendrocyte loss localised to Aβ plaques

In all PS-1 familial, sporadic and preclinical AD cases and transgenic AD models, Aβ plaques were associated with a focal reduction in FluoroMyelin-labelled profiles (Figure 6.1 A, B; Figure 6.2 A, B). In contrast, diffuse Aβ deposits were not associated with demyelination (Figure 6.2 C, D). In PS-1 familial, sporadic and preclinical AD cases, there was a significant decrease (60.2 ± 4.1%, 56.6 ± 5.2% and 35.3 ± 3.3% respectively, mean ± SEM; $p < 0.01$) in plaque-associated myelinated axons compared to equivalent areas in age-matched controls (Figure 6.3 A, C). Furthermore, in sporadic but not preclinical AD cases, there was a significant loss of oligodendrocytes (59.4 ± 3.7%, $p < 0.01$) at the edge of Aβ plaques (Figure 6.4 G, H). Both Tg2576 (57.6 ± 5.7%) and APP/PS1 (46.0 ± 5.2%) transgenic mice exhibited fewer myelinated axons near Aβ plaques ($p < 0.01$; Figure 6.3 B, D) compared to similar plaque-free areas in controls. Abundant plaques in the superficial subcortical white matter of Tg2576 mice were also associated with focal demyelination (Figure 6.1 D, E) but were not included in the quantitative analysis. Interestingly, plaque-free cortical grey matter (>100μm from nearest plaque, layer 5) of both human AD and transgenic mouse sections showed no significant loss of myelin relative to control tissue (Figure 6.3 A, B). Similarly, there was no significant loss of oligodendrocytes in plaque-free areas of sporadic and preclinical AD cases compared to controls.

Gallyas staining for myelin also showed focal demyelination consistent with fluorescent labelling (Figure 6.1 G, H). Furthermore, double labelling demonstrated demyelination of SMI312-positive axons (Figure 6.1 I), which were invariably

Figure 6.1

Immunolabelling for Aβ (blue) demonstrates focal demyelination localized to plaques in cortical layer 5 in humans (A, B), as well as in white matter regions of Tg2576 transgenic mice (arrows; D, E). A sample of the quantitation method (C, same region as B but inverted) is shown with templates corresponding to 50%(1), 100%(2) and 150%(3) of the plaque perimeter of the plaque shown in (A). Control human cases demonstrate myelinated axons double-labelled with Fluoromyelin™ (green) and the pan-axonal antibody marker SMI312 (red) (arrowheads; F), whereas a similar cortical area in an end-stage AD brain (I) shows demyelinated axons (arrows) in proximity to an Aβ plaque (asterisk). A modified Gallyas stain for myelin demonstrated numerous 'holes' in staining in human AD (arrows, G) and transgenic brain sections, which at higher magnification, corresponded to lower myelin content (H, inset from G).

Scale bar = 50μm (A, B, C, H, I); 20μm (F); 100μm (D, E); 200μm (G)

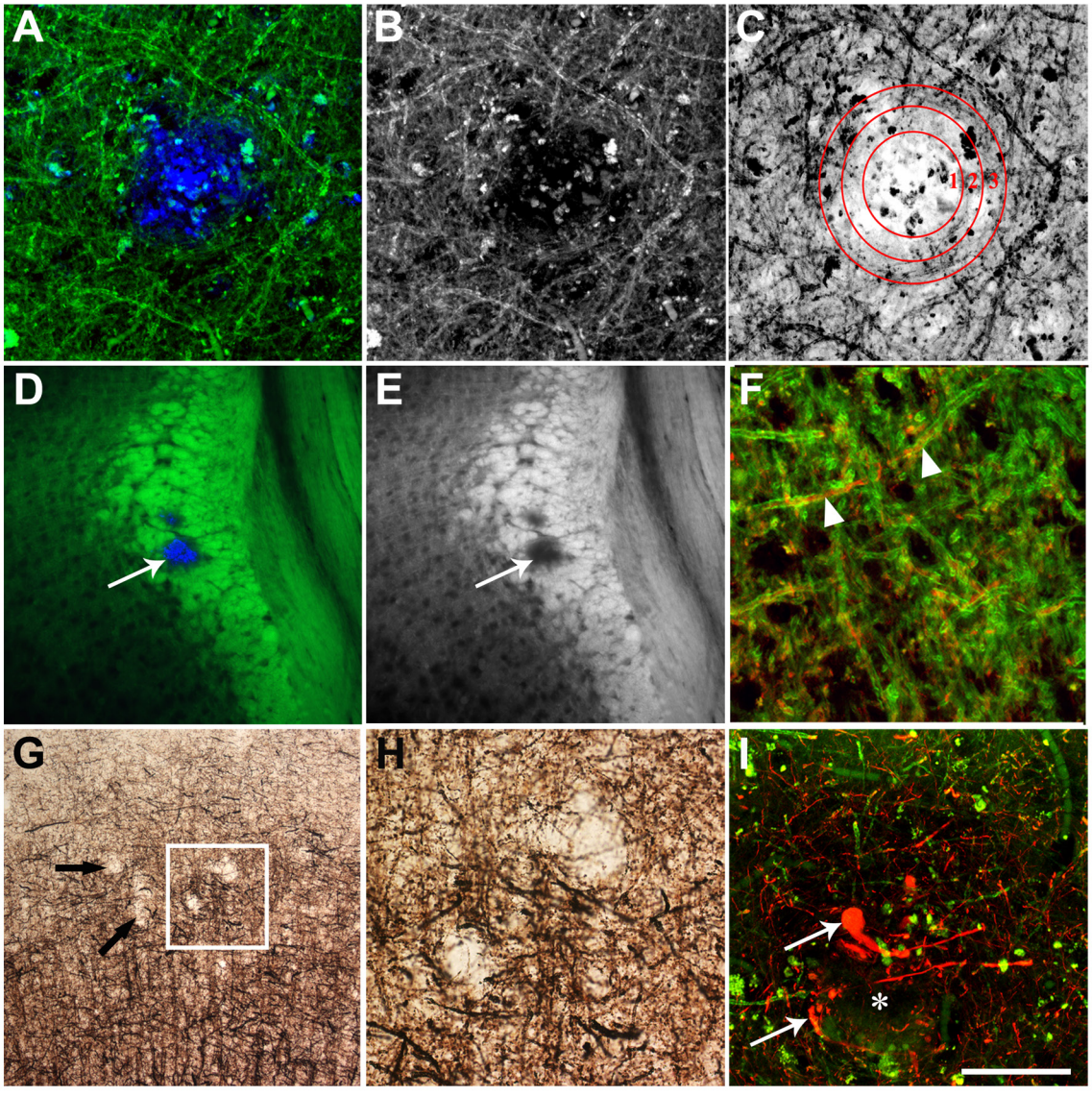

A
B
C
1
2
3
D
E
F
G
H
I
*

Figure 6.2

Immunolabelling with rabbit pan-β-amyloid (blue) and Fluoromyelin (green) in a PS1 familial AD case shows extensive demyelination around and within a 'cotton-wool' morphology Aβ plaque (A, B) however, this was not observed in a diffuse morphology Aβ deposit (C, D). Scale bar = 50μm

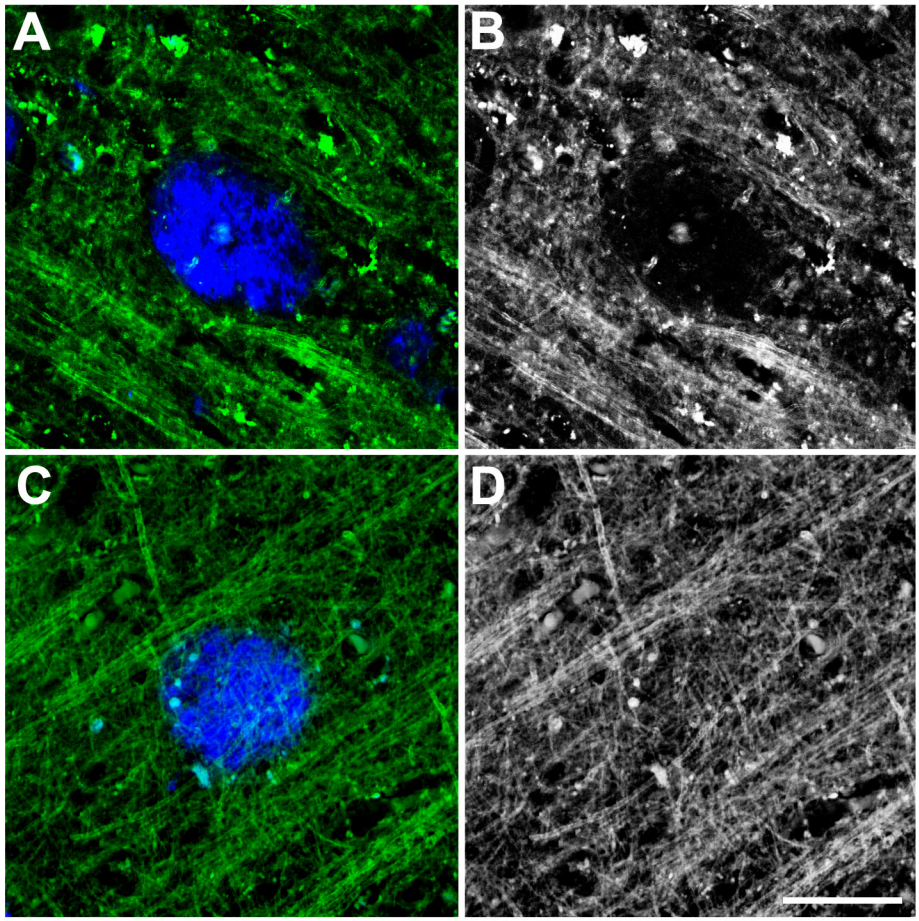
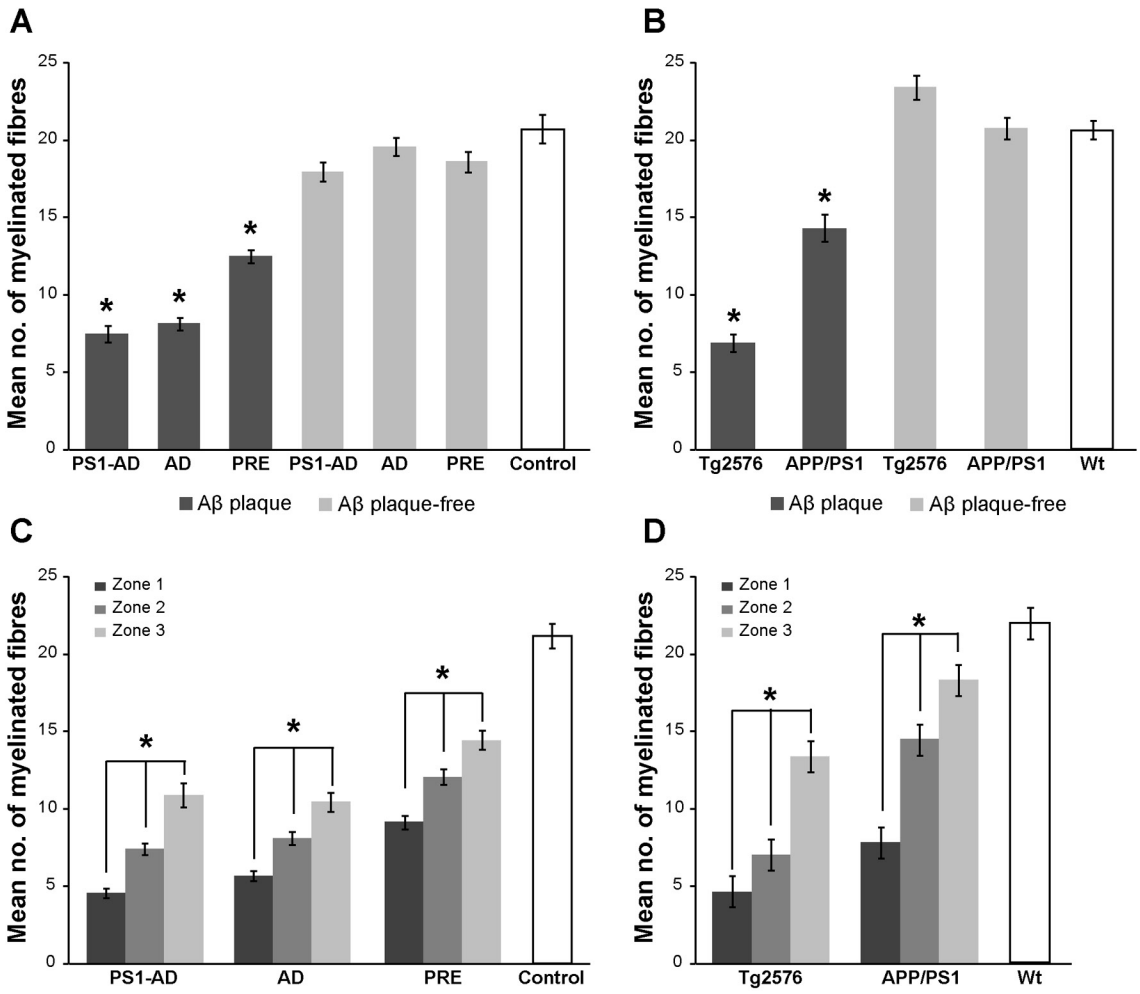

A
B
C
D

Figure 6.3

There was a significant difference in the number of myelinated axons intersecting the plaque edge (100% of plaque area) in plaque-containing and plaque-free regions in PS-1, sporadic and preclinical AD cases compared to healthy controls (A). In both transgenic mice models, only the plaque-containing regions had significantly less myelinated axons compared to wildtype mice (B). The plaque-core region (50% area) displayed the highest degree of myelin-loss compared to the plaque-edge (100% area) and the plaque-periphery (150% area) in both human cases (C) and in mice (D). (* $p < 0.05$, Dunnett's post hoc test)


A
Mean no. of myelinated fibres
25
20
15
10
5
0
PS1-AD
AD
PRE
PS1-AD
AD
PRE
Control
Aβ plaque
Aβ plaque-free
B
Mean no. of myelinated fibres
Tg2576
APP/PS1
Tg2576
APP/PS1
Wt
Aβ plaque
Aβ plaque-free
C
Mean no. of myelinated fibres
Zone 1
Zone 2
Zone 3
PS1-AD
AD
PRE
Control
D
Mean no. of myelinated fibres
Zone 1
Zone 2
Zone 3
Tg2576
APP/PS1
Wt

myelinated in the surrounding neuropil and age-matched control tissue (Figure 6.1 F).

Demyelination concentrated at the plaque core

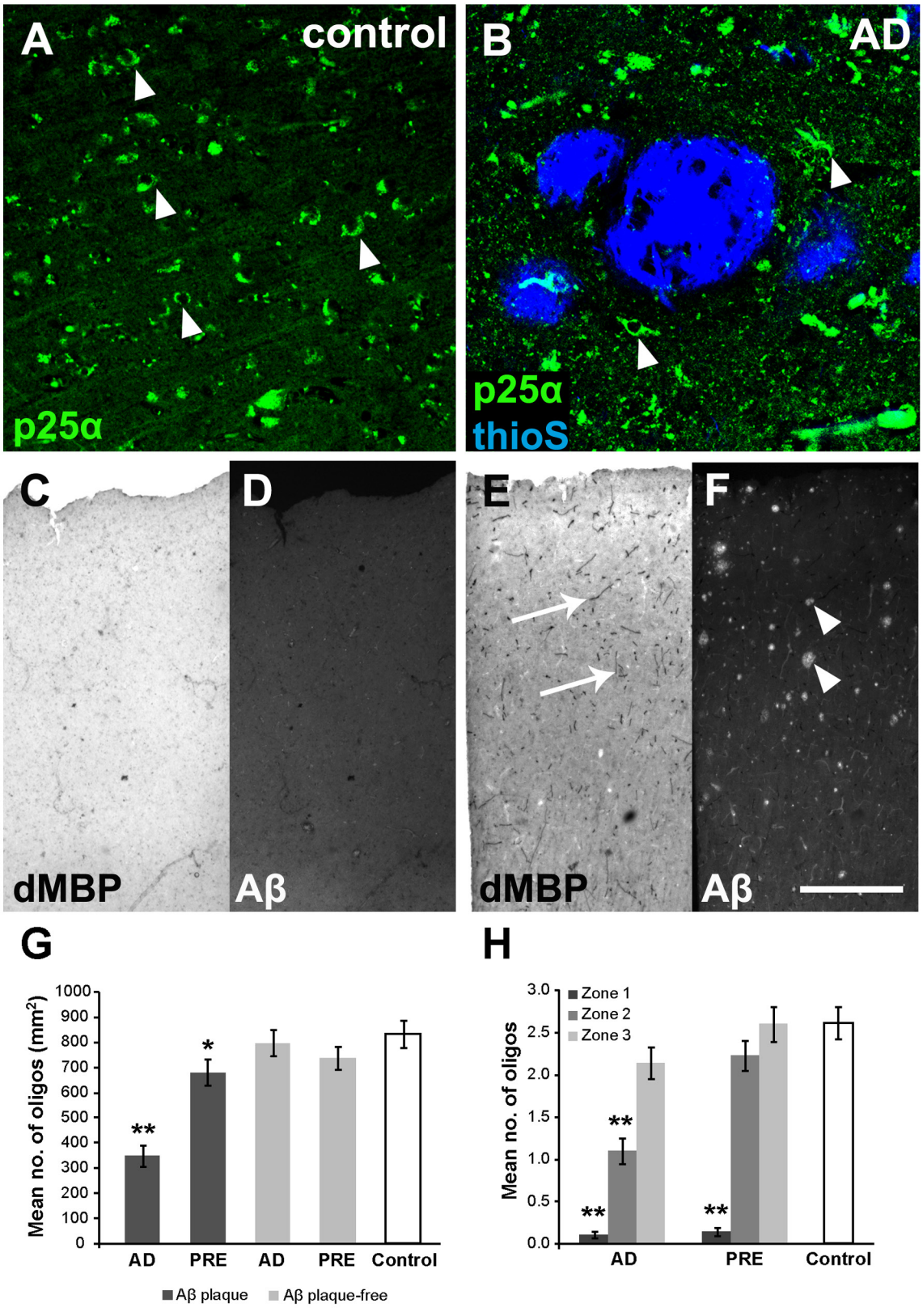
To ascertain if demyelination and oligodendrocyte loss occurred uniformly across Aβ plaque substructure, I compared myelin loss at the core to the edge and to the immediate plaque periphery, as defined in the Methods section. The greatest effect seen in human cases occurred at the plaque core, with up to 78 ± 5.7% loss for PS-1 familial AD cases, 72 ± 5.3% in sporadic AD and 57.6 ± 5.8% in preclinical AD, compared to control tissue ($p < 0.01$). A similar pattern of myelin loss occurred in both lines of transgenic mice, with up to 71 ± 5.1% loss at the plaque-core in Tg2576 tissue and 60 ± 5.9% in APP/PS1, compared to wildtype animals ($p < 0.01$). This effect decreased with increasing distance from the plaque-core in both the human and transgenic mouse cases (Figure 6.3 C, D). Interestingly, significant deficits in myelination relative to control values were evident even in the peripheral region surrounding the plaque. In the human cases, there was a 46 ± 5.4% and 48 ± 7.6% decrease in the number of myelinated axons in PS1 familial and sporadic AD cases respectively, while there was a 28 ± 8.3% decrease in preclinical AD, compared to control tissue (Figure 6.3 C; $p < 0.01$). In transgenic mice, a similar peripheral zonal plaque-associated myelination decrease was demonstrated, with a 57 ± 9.7% loss of myelinated fibres in Tg2576 cases and 46 ± 7.6% in APP/PS1 cases compared to wildtype controls (Figure 6.3 D; $p < 0.01$).

There was virtually complete loss of oligodendrocytes at the plaque core in both sporadic end-stage and preclinical AD cases (96.7 ± 1.2% and 96.2 ± 1.8% loss; Figure 6.4 B, H), as well as considerable loss at the plaque edge in sporadic AD

Figure 6.4

Immunolabelling with rabbit anti-p25-α (green) and rabbit anti-pan-β-amyloid (blue) in human control (A) and sporadic AD (B) cases shows loss of oligodendrocytes (arrowheads, A, B) at the plaque edge and core, but not in the periphery. DAB staining with rabbit anti-degraded MBP (arrows, C and E) and fluorescent labelling with mouse anti-pan-β-amyloid (D and F; arrowheads indicate Aβ plaques) shows accumulation of degraded MBP in AD cases (E), but not in human controls (C). There was a significant reduction in oligodendrocyte numbers in Aβ plaque-rich neuropil in sporadic AD cases (G), as well as in the plaque-core in both preclinical and sporadic AD cases (H) compared to plaque-free and control regions.

Scale bar = 50μm (A, B); 400μm (C–F)


A
control
p25α
B
AD
p25α
thioS
C
dMBP
D
Aβ
E
dMBP
F
Aβ
G
Mean no. of oligos (mm²)
1000
900
800
700
600
500
400
300
200
100
0
**
*
AD
PRE
AD
PRE
Control
Aβ plaque
Aβ plaque-free
H
Mean no. of oligos
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Zone 1
Zone 2
Zone 3
**
**
AD
PRE
Control

cases (59.4 ± 3.7% loss, $p < 0.01$). There was no significant loss of oligodendrocytes at the plaque edge in preclinical AD, and both sporadic and preclinical AD cases showed no oligodendrocyte loss at the plaque periphery compared to controls. A degenerated MBP (dMBP) antibody that only recognizes demyelinated tissue, showed more staining in AD cases around Aβ plaques and did not appear to stain control cortical tissue (Figure 6.4 C-F).

Presence of CAA did not affect degree of demyelination or oligodendrocyte loss

To exclude CAA as a possible cause of cortical demyelination in the cases examined, the type and severity of CAA were assessed for individual cases. Depending on the type of vessels affected, CAA can be differentiated into Type 1, which includes cerebral and leptomeningeal arteries, arterioles and capillaries, or Type 2, which only affects larger vessels and has no capillary involvement (Thal et al. 2002). Virtually all of the sporadic cases used in this study displayed type 1 CAA of varying severity, while only two PS-1 familial and one preclinical AD cases of mild severity displayed type 1 and type 2 CAA respectively (Table 6.1; Figure 6.5 A-C). When compared to the degree of demyelination ($p = 0.09$) or oligodendrocyte loss ($p = 0.14$) in the respective cases, the type and severity of CAA did not have any notable effects (one-way ANOVA).

Reduced number of mature oligodendrocytes and increased number of OPCs in young and old APP/PS1 mice are indicative of cortical demyelination with a concurrent remyelinating response

Oligodendrocyte lineage cells were labelled with markers for different states of maturity: PDGFRα for OPCs (Figure 6.6 I; Rivers et al. 2008), GPR17 for immature, pre-myelinating oligodendrocytes (Figure 6.6 J; Chen et al. 2009) and CC-1 for

Figure 6.5

Sections were immunolabled with mouse anti-pan-β-amyloid (red) and rabbit anti-desmin (green) to determine the type and severity of CAA present. Vessel diameter and absence of desmin-labelled smooth muscle (arrowheads) was used to determine capillary (arrow, A) or arteriole (B) CAA involvement. In CAA, Aβ accumulates in the basal membrane of capillaries (arrow, A) and/or adventitia (arrowheads, B) and lumen (C) of larger vessels.

Scale bar = 20μm (A); 50μm (B); 80μm (C)

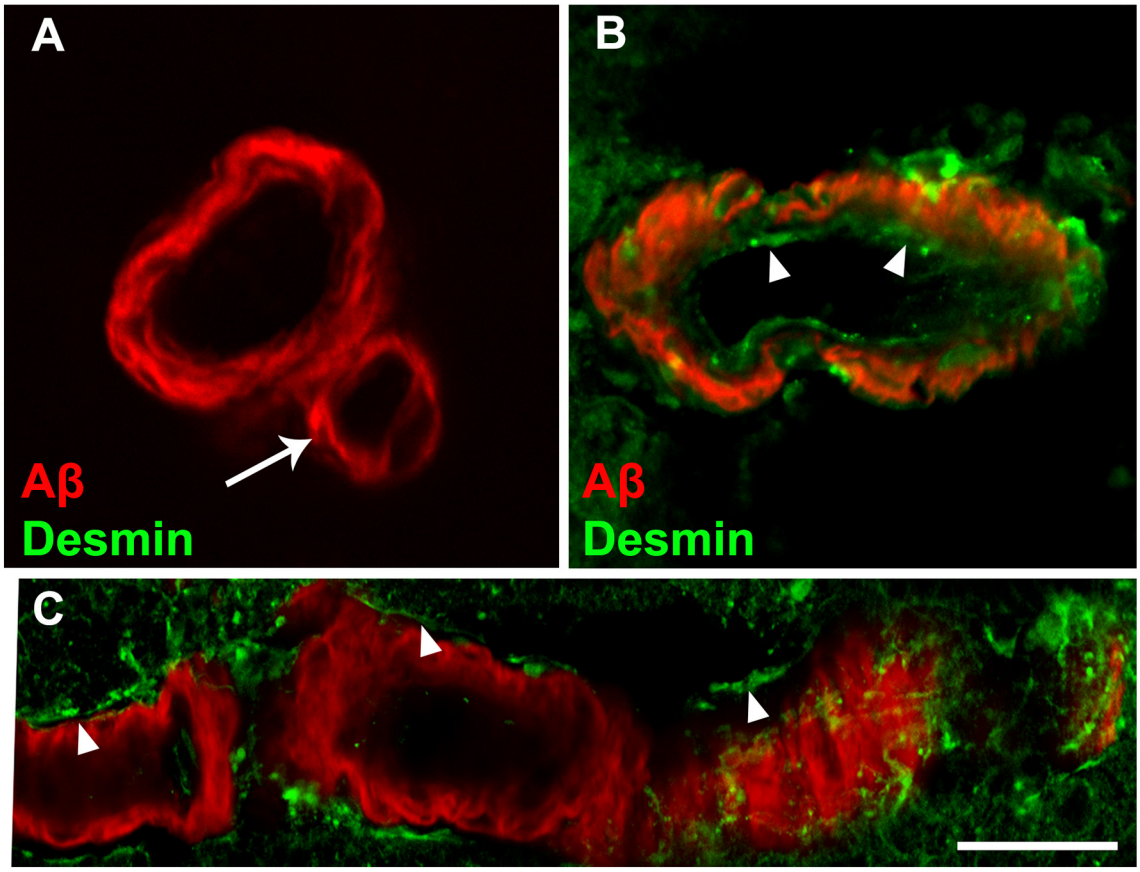
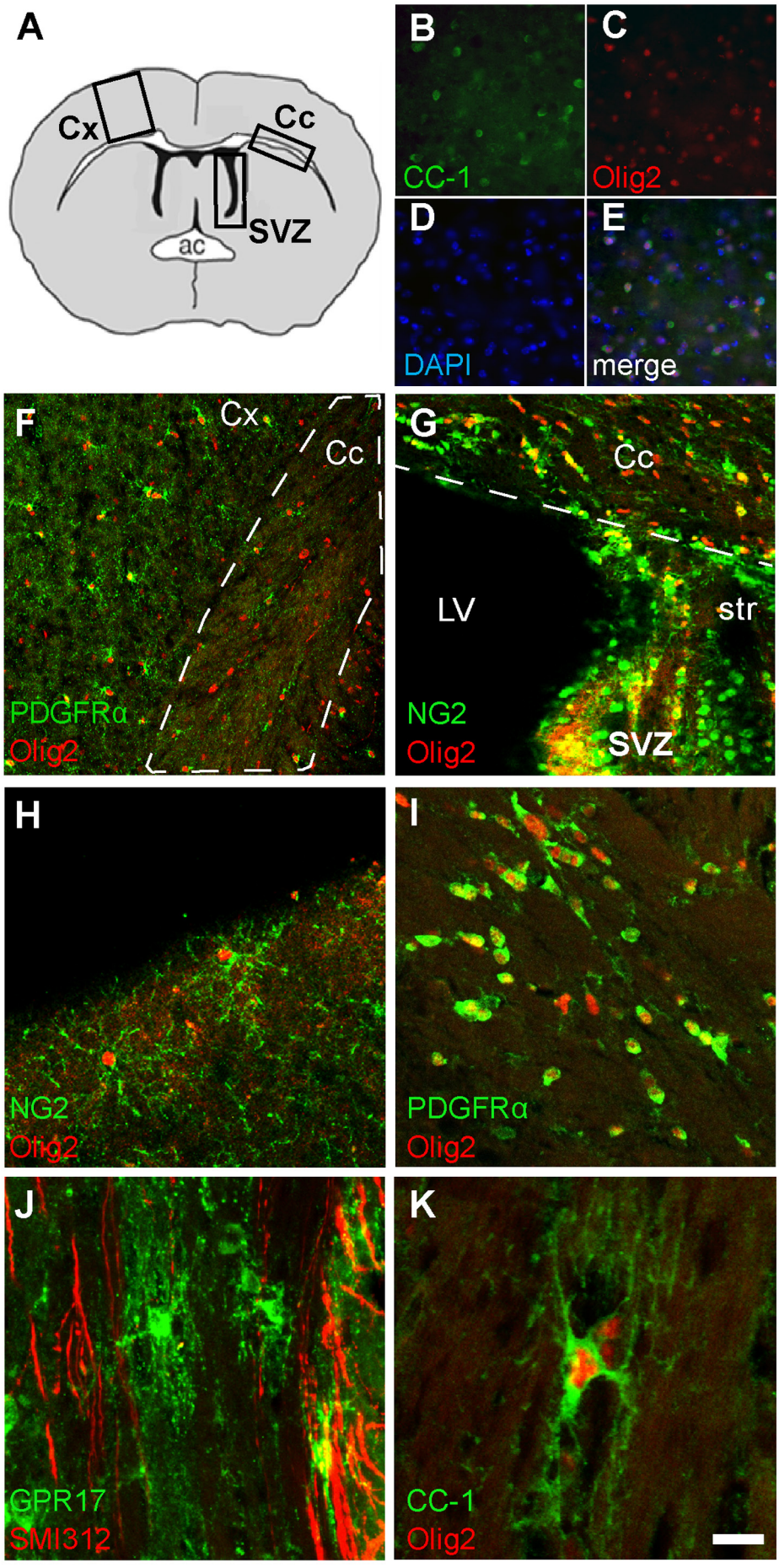

A
Aβ
Desmin
B
Aβ
Desmin
C

Figure 6.6

Coronal sections from APP/PS1 transgenic mice were processed as previously described and regions from the somatosensory (S1) cortex (Cx, F), corpus callosum (Cc, F) and subventricular zone (SVZ, G) were chosen for analysis (illustrated in A). Sections were counterlabelled with pan-oligodendrocyte lineage transcription factor Olig2 (C) and DAPI (D) to ensure only viable oligodendrocyte cells were analysed (B–E). Example sections from APP/PS1 tissue labelled with NG2 (H), PDGFRα (I), GPR17 (J) and CC-1 (K) are shown. Scale bar = 10μm (J, K), 50μm (F–I), and 100μm (B–E)


A
Cx
Cc
SVZ
ac
B
CC-1
C
Olig2
D
DAPI
E
merge
F
Cx
Cc
PDGFRα
Olig2
G
Cc
LV
str
NG2
Olig2
SVZ
H
NG2
Olig2
I
PDGFRα
Olig2
J
GPR17
SMI312
K
CC-1
Olig2

mature, myelinating adult oligodendrocytes (Figure 6.6 K; Dimou et al. 2008). There were significantly more OPCs in six and twelve month-old APP/PS1 mouse cortex (Figure 6.7 A; $p < 0.001$; 153 ± 8.1 and 146 ± 7.6 versus 111 ± 2.9 and 79 ± 9.1 cells/mm^2 respectively), whereas in the corpus callosum, there were significantly more OPCs only in twelve month-old in APP/PS1 mice (Figure 6.7 B; $p < 0.001$; 215 ± 5.4 versus 144 ± 8.9 cells/mm^2). For pre-myelinating immature oligodendrocytes, there were significantly more cells in the twelve month-old APP/PS1 cortex only (Figure 6.7 C, D; $p < 0.001$; 175 ± 17.6 versus 86 ± 12.3 cells/mm^2). There were significantly fewer CC1-immunolabeled cells in six and twelve month-old APP/PS1 mouse cortex compared to wildtype littermates, however there was no difference in the corpus callosum (Figure 6.7 E, F; $p < 0.01$; 150 ± 9.0 and 210 ± 18.6 versus 193 ± 10.4 and 294 ± 24.5 cells/mm^2). Finally, in the SVZ, there was almost a doubling in the number of NG2-immunopositive OPCs in twelve month-old APP/PS1 mice compared to controls (Figure 6.7 G; $p < 0.001$; 198 ± 23.0 versus 110 ± 9.3 cells/mm^2).

Altered levels of myelin-associated proteins in young and old APP/PS1 mice

Samples of purified myelin were extracted from three, six and twelve month-old transgenic and wildtype mice and immunoblotted for four key myelin proteins (Figure 6.8 A). The relative abundance of CNPase and MBP were significantly decreased in purified myelin samples from twelve month-old APP/PS1 mice, while the levels of PLP were significantly lower in both six and twelve month-old transgenic animals compared to controls (Figure 6.8 B, C, D; $p < 0.05$). Interestingly, there was an increase in the abundance of MAG protein from six and twelve month-old transgenic samples compared to controls (Figure 6.8 E; $p < 0.05$).

Figure 6.7

Graphs illustrating the numbers of oligodendrocyte precursor cells (A, B, G), immature oligodendrocytes (C, D) and mature myelinating oligodendrocytes (E, F) in APP/PS1 and wildtype cortex. There were significantly more OPCs in both six and twelve month-old cortex (Cx; A), as well as in twelve month-old corpus callosum (Cc; B). Similarly, there were significantly more NG2 cells in the twelve month-old transgenic SVZ (G), while GPR17-labelled immature oligodendrocytes were more numerous in twelve month-old transgenic cortex (C, D). In contrast, there were fewer CC-1-immunolabelled mature oligodendrocytes in APP/PS1 cortex (E, F). Error bars denote standard error of the mean. (* $p < 0.01$, ** $p < 0.001$, Student's *t*-test)

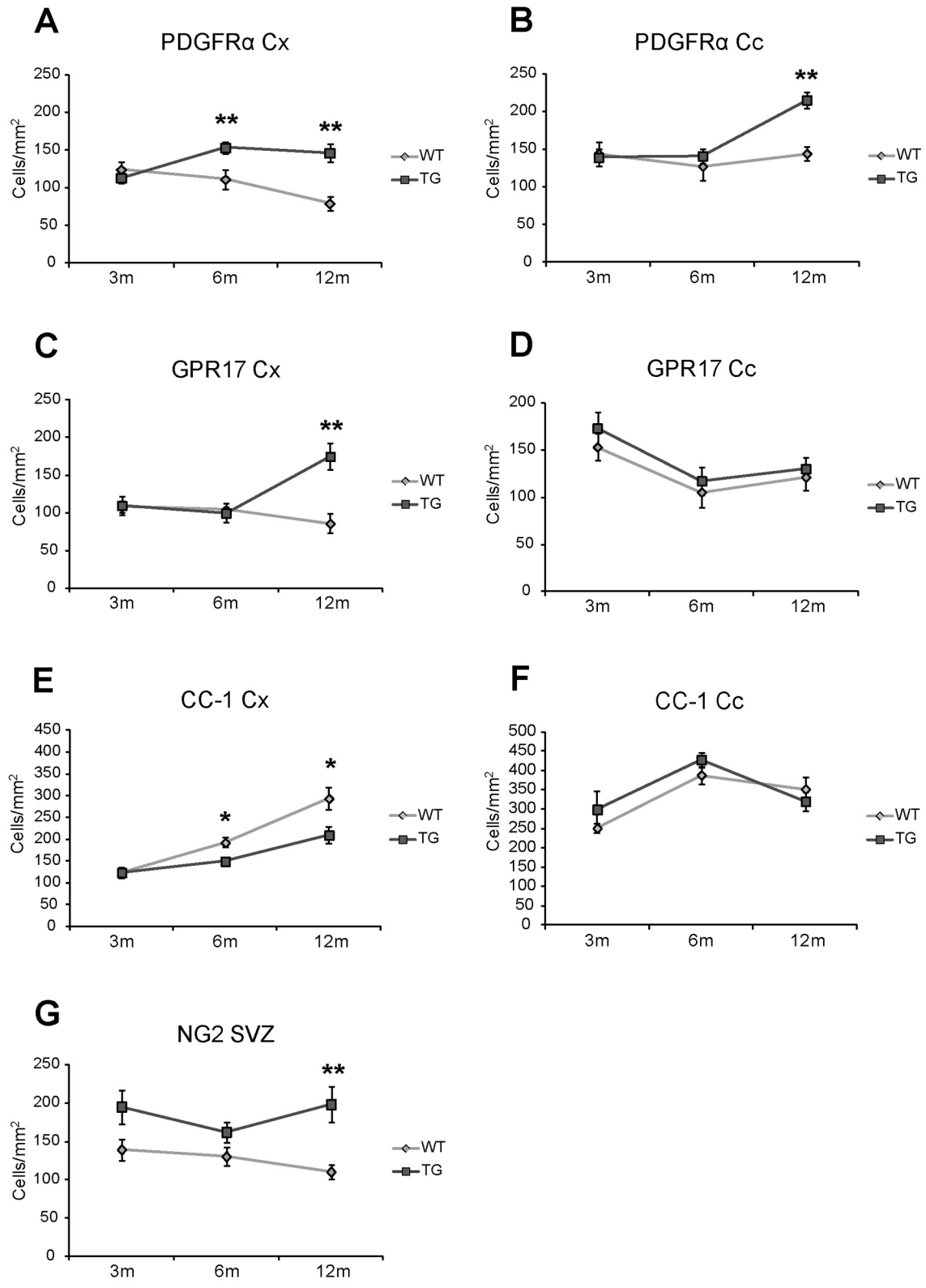
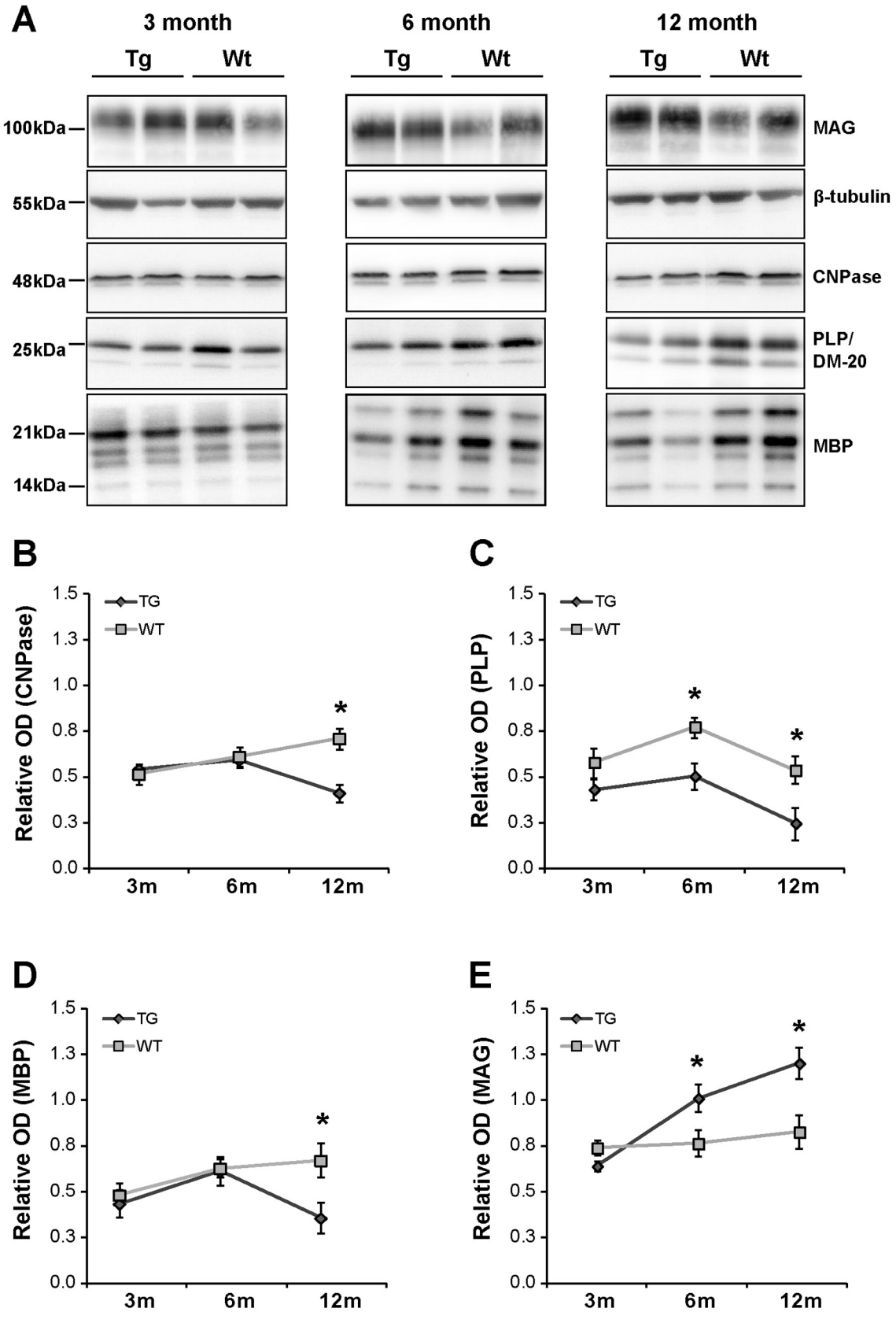

A
PDGFRα Cx
Cells/mm2
250
200
150
100
50
0
3m
6m
12m
**
**
WT
TG
B
PDGFRα Cc
**
C
GPR17 Cx
**
D
GPR17 Cc
E
CC-1 Cx
450
400
350
300
250
200
150
100
50
*
*
F
CC-1 Cc
500
G
NG2 SVZ
**

Figure 6.8

Purified myelin samples from three, six and twelve month old APP/PS1 mice and littermate controls (n = 5 each) were collected and immunoblotted with a range of myelin-associated proteins (A). There was a decrease in CNPase (B), PLP (C) and MBP (D) protein levels in twelve month-old transgenic animals. (E) The levels of MAG protein were significantly higher in six and twelve month-old APP/PS1 mice. Error bars denote standard error of the mean. (* $p < 0.05$, Student's *t*-test)


A
3 month
6 month
12 month
Tg
Wt
100kDa
55kDa
48kDa
25kDa
21kDa
14kDa
MAG
β-tubulin
CNPase
PLP/
DM-20
MBP
B
Relative OD (CNPase)
C
Relative OD (PLP)
D
Relative OD (MBP)
E
Relative OD (MAG)
TG
WT
1.5
1.3
1.0
0.8
0.5
0.3
0.0
3m
6m
12m
*

6.4 Discussion

These data demonstrate a novel finding of focal demyelination associated with fibrillar Aβ plaques in AD and related experimental models, but not with more diffuse Aβ deposits or in plaque-free areas. Plaque-associated dystrophic neurites labelled with axonal markers were also demyelinated. Additionally, there was a plaque-associated loss of mature oligodendrocytes in human cases. In mice, the overall number of mature oligodendrocytes was also significantly reduced, while the number of OPCs and immature non-myelinating oligodendrocytes was higher than littermate controls. Interestingly, the number of NG2-expressing OPC cells in the SVZ in APP/PS1 mice nearly doubled compared to control mice. In addition to OPCs residing in the corpus callosum and parts of the cortex, the SVZ is a source of adult-born OPCs (Gonzalez-Perez and Alvarez-Buylla 2011; Morrens et al. 2012) that can be stimualated to differentiate into new oligodendrocyte during remyelination (Kang et al. 2010; Tripathi et al. 2010). Furthermore, integral myelin-associated proteins were found to be reduced in aged, plaque-bearing transgenic mice compared to controls. In concert with perturbations of dendritic spines and synapses (Spires et al. 2005; Koffie et al. 2009), such focal disruption of myelination is likely to contribute to deficits in information processing within and between cortical modules.

It is well established that white matter integrity progressively deteriorates with normal aging (Meier-Ruge et al. 1992; Tang et al. 1997), but evidence from gross brain imaging studies suggests that this natural tendency could be exacerbated and accelerated in AD (de la Monte 1989; Bartzokis et al. 2003; Stricker et al. 2009). In this regard, the data from the presenilin-1 familial AD cases represents a valuable subset of AD cases as the age-of-onset is usually much earlier compared to sporadic

AD cases, and as such offers a potential glimpse of the action of Aβ on nearby myelin before age-related and vascular confounding effects have fully developed. Furthermore, although white matter lesions are present in both AD and non-demented aging, it has been proposed that, in AD, such lesions may influence the severity of cognitive impairment, especially in the earlier stages of the disease (Burns et al. 2005). The aetiology of subcortical demyelination remains controversial: diffuse white matter lesions have been reported even in the early stages of AD independent of any signs of infarction, white matter amyloid angiopathy, or Wallerian degeneration (Brun and Englund 1986; de la Monte 1989; Bartzokis et al. 2003); whereas strong evidence links age-related ischemic insults and overall deterioration of cerebrovascular function to white matter damage (Brown et al. 2000; Haglund and Englund 2000; Tian et al. 2004). It is likely that the demyelination processes involved may be different depending on the key underlying pathology present (Ihara et al. 2010). It is important to note, however, that there is no direct correlation between the severity of white matter lesions with the overall degree of cortical AD progression (Sjobeck et al 2006), so the demyelination observed here could be a separate process not immediately related to subcortical white matter lesions. Furthermore, the type and relative severity of concomitant CAA in the current samples did not appear to affect the degree of cortical demyelination or oligodendrocyte loss in this study.

It is not clear whether plaque-associated demyelination represents a primary event related to Aβ deposition or whether it may follow secondary alterations in axons that lead to dystrophic neurite formation. There is certainly evidence for both. On the one hand, changes in WM integrity occur very early in AD progression, with reports of altered functional anisotropy and axial diffusivity in MCI patients (Huang and

Auchus 2007; Kavcic et al. 2008; Salat et al. 2010; Gold et al. 2012; Huang et al. 2012; also see section 1.6.4). This is backed up by similar findings in Tg2576 and APP/PS1 AD mice (Song et al. 2004; Sun et al. 2005; Chen et al. 2011b; Zerbi et al. 2012) which found axonal loss correlated well with myelin perturbations. This view that alteration in myelin composition and/or its loss lead to axonal dysfunction and eventual plaque deposition has been adopted as some to be one of the primary events in AD pathogenesis (Bartzokis 2004, 2011). Further support for this idea comes from the finding that the most sever changes in myelin occur in later-myelinating tracts/regions of the temporal and frontal cortex– regions which are classically implicated in being the earliest sites of Aβ pathology in AD (Reisberg et al. 1999; Braak et al. 2000; Bartzokis 2004; Stricker et al. 2009; Fornari et al. 2010). Could myelin pathology precede and/or lead to Aβ deposition in these regions as a result of axonal dysfunction? This is a very intriguing possibility indeed and warrant further examination. On the other hand, age-related loss of myelin occurs independently of Aβ deposition in healthy controls (Lindner et al. 2009; Stricker et al. 2009; Bartzokis et al. 2012; Lu et al. 2013), raising the question of how much of early AD/MCI myelin abnormalities are due to ageing and not part of AD pathogenesis per se? Furthermore, there is also evidence that FAD mutations (Ringman et al. 2007; Stein et al. 2012) as well as carrying the AD risk factor APOEε4 allele (Bartzokis et al. 2007; Lu et al. 2011), predispose and potentiate the observed myelin phenotype further implying that myelin pathology may be downstream of Aβ toxicity.

The present data suggest that there is significant demyelination occurring in preclinical AD, before overt Aβ plaque-associated oligodendrocyte loss occurs as seen in sporadic AD cases. This is in line with recent evidence showing white matter

disruption occurring early in preclinical and presymptomatic FAD mutation carriers (Ringman et al. 2007). In the present study, immunoblots from purified myelin samples demonstrated a reduction in myelin basic protein, CNPase and proteolipid protein in APP/PS1 transgenic mice compared to wildtype littermate controls, consistent with previous reports (Roher et al. 2002; Desai et al. 2009). Interestingly, there was a robust increase in myelin associated glycoprotein (MAG) levels in both six and twelve month-old transgenic samples. One potential explanation for this counterintuitive increase is that MAG has recently been found to be neuroprotective post-axonal injury (Nguyen et al. 2009), therefore its higher expression in transgenic samples could signify a protective response. Therefore, alterations in myelin makeup, and thus, potentially quality/function, may be a very early event in AD pathogenesis, occurring in presymptomatic cases and young (6month) APP/PS1 mice.

Damaged cortical myelin is actively removed and replaced, a dynamic process that has been shown to continue well into old age, as evidenced by a doubling of intracortical oligodendrocytes in the aged primate brain (Peters et al. 2008). Therefore, ongoing remyelination and compensation from less affected brain regions could presumably limit the cognitive impact of focal demyelination. Remyelination has also been reported in end-stage cortical grey matter lesions of both chronic human MS cases (Kutzelnigg et al. 2005) and rodent MS models (Merkler et al. 2006), in which it appeared to compensate for some of the ongoing demyelination at lesion sites, resolving associated inflammation more quickly than in similar lesions in the white matter (Merkler et al. 2006; Albert et al. 2007). This mild degree and rapid resolution of cortical demyelination contrasts with the more extensive damage observed in this study, which may represent different pathophysiological pathways, or chronic effects of Aβ plaques on the surrounding neuropil, including aberrant

regenerative/sprouting responses of demyelinated axons. This may impair remyelination attempts locally, exacerbating age- and region-associated declines in remyelination capacity (Shen et al. 2008) and predisposing neurons to apoptosis (Irvine and Blakemore 2008).

Although there was no significant oligodendrocyte loss in the plaque periphery and plaque-free regions of preclinical and sporadic AD cases, there was a significant Aβ plaque-associated reduction in oligodendrocytes numbers in the latter, as well as virtually complete loss inside the plaque core in both case types. Local oligodendrocyte depletion by possible Aβ toxicity, combined with the increased expression of dMBP in AD cases compared to controls, suggests a true degenerative change in oligodendrocytes and myelin, rather than a transient and probably reparable change as part of the myelination/remyelination process. This is quite likely considering Aβ toxicity to oligodendrocytes (Xu et al. 2001; Lee et al. 2004; Roth et al. 2005; Chen et al. 2006) but not OPCs (Horiuchi et al. 2012) observed *in vitro,* and the fact that oligodendrocytes residing in the grey matter may be more vulnerable to metabolic disturbances than ones in the white matter (Bauer et al. 2002). A recent study by Behrendt et al (2013) has also shown early oligodendrocyte loss *in vivo* in APP/PS1 mice and AD cases with a concomitant increae in OPCs similar to the present findings.

This suggest that there is a complex relationship between intrinsic myelin integrity (cf. Bartzokis 2004) and Aβ plaque build-up in mediating axonal dysfunction, as opposed to a simple linear/causal relationship. It is quite likely that both act synergistically to bring about cortical dysfunction in AD (Jack et al. 2009; Tosun et al. 2011; Bartzokis 2011). In this respect, recent studies have shown that Aβ can

mediate gliotransmission and transcriptional changes in astrocytes at physiologically relevan concentrations (Liu et al. 2013). Given that oligodendrocytes also express functional α7-nAChRs (Velez-Fort et al. 2009) and M1-5 mAChRs (DeAngelis et al. 2012), it is highly likely that Aβ may also modulate oligodendrocyte/OPC function both physiologically, as well as during its pathological build-up in AD.

Therefore, despite ongoing remyelination, Aβ plaques could lead to focal demyelination and axonal damage as seen in chronic multiple sclerosis lesions (Lindner et al. 2009). Furthermore, the apparent persistence of these demyelinating zones around plaques suggests impairment of compensatory mechanisms normally triggered by demyelination (Fancy et al. 2010) by some aspect of the plaque environment. In particular, the lack of oligodendrocytes in these regions in sporadic AD cases suggests that differentiation of oligodendrocyte precursors may be somehow impaired. In support of this, this study showed an early and significant increase in OPC and immature oligodendrocyte cell numbers in APP/PS1 transgenic mice that was accompanied by a selective decrease in mature CC1-positive oligodendrocytes in the Aβ plaque-rich cortex.

In conclusion, this study demonstrates a novel finding of focal demyelination exclusively associated with Aβ plaques, indicating a focal disruption to cortical circuitry. Such focal disruption in neuronal connectivity is likely to contribute to deficits in cortical processing. Normal compact myelin is essential for maturation of the neuronal cytoskeleton and proper neuronal function (Brady et al. 1999), therefore its loss in AD, particularly around Aβ plaques, may be an important pathological process that could be involved both in disease pathogenesis, as well as affecting regenerative/repair processes.

7. Discussion

Alzheimer's disease is the most common form of dementia, affecting an estimated 36 million people worldwide (Mayeux and Stern 2012; Sosa-Oritz et al. 2012). Due to improvements in geriatric care and the ageing population in developed countries, this figure is predicted to rise to 65 million by 2030, creating a significant social and economic burden (estimated global cost of AD in 2010: US$600 billion; World Alzheimer's Report 2010). Despite considerable efforts, a complete appreciation of the causes and mechanisms that drive AD, as well as effective treatment strategies and interventions, still elude us (Huang and Mucke 2012). Confounding matters further is the fact that many of the pathological changes associated with AD, particularly at earlier stages, are also part of normal cognitive aging (Honer et al. 2012; Morrison and Baxter 2012).

Although widespread neuronal degeneration and eventual death are the inevitable outcome of AD, cognitive symptoms manifest much earlier during the prodromal phase that likely begins decades prior. This suggests that cellular dysfunction, as opposed to overt cell death, is the more likely substrate for cognitive decline. In this regard, the well-conserved regional and temporal pattern of AD pathology progression across cases suggests a 'path of least resistance' that could signify a graded susceptibility based on brain region (Gsell 2004; Braak et al. 2011). Selective vulnerability could also manifest at the cellular level, with specific neuronal and glial populations being differentially affected by AD pathology. Elucidating cell-type-specific effects of AD pathology at early stages of the disease would not only help consolidate our current understating of disease mechanisms, but also potentially identify novel therapeutic targets. Therefore, the central aim of this thesis was to

investigate how particular neuronal and glial populations are affected by AD progression, and whether compensatory mechanisms are recruited in response.

With regards to neuronal vulnerability to AD pathology, previous work has shown that pyramidal cells in the entorhinal cortex and dentate gyrus of the hippocampus as well as cortical association areas in the temporal and frontal cortices are particularly susceptible to developing NFT and DN pathology in AD (Hyman et al. 1984, 1990; Lewis et al. 1987; Hof and Morrison 1990a,b; Gomez-Isla et al. 1996; Hof et al. 1999; Vickers et al. 2000; Morrison and Hof 2007; Stranahan and Mattson 2010). Interestingly, this population of vulnerable pyramidal neurons has considerable overlap with the subpopulation expressing the NF-triplet family of intermediate filaments (Morrison et al. 1987; Hof et al. 1990; Hof and Morrison 1990; Vickers et al. 1992, 1994; Hof et al. 1995; Mann et al. 1996). Non-pyramidal interneurons on the other hand, which largely do not express NFs, have previously been shown to be relatively spared in AD (Hof et al. 1991, 1993; Fonseca and Soriano 1995; Sampson et al. 1997; Leuba et al. 1998; Mikkonen et al. 1999; Pugliese et al. 2004). More recently however, this assumption has been questioned by conflicting reports of interneuron-specific deficits and degeneration in vulnerable regions such as the hippocampus (see Chapter 1.4, Koliastos et al. 2006; Baglietto-Vargas et al. 2010; Takahashi et al. 2010). Therefore, to address this contentious issue, this thesis morphologically analysed NF- and CR-immunoreactive neurites in human preclinical, end-stage and control tissue, as well as in two widely used transgenic AD models. Specifically, the effect of proximity to Aβ deposits on cortical neurite morphology was investigated, with the hypothesis that NF-ir but not CR-ir neurites will be more likely to develop DNs.

As expected, Aβ plaques were particularly disruptive to NF-ir neurites traversing the plaque core and edge, leading to a large proportion developing DN morphology, whereas far fewer CR-ir neurites at the plaque edge were dystrophic. Interestingly, unlike NF-ir neurites' often bulb-like or stubby appearance at the plaque edge, CR-ir processes often appeared to traverse around plaques. Similarly, although the degree of DN formation correlated strongly with plaque size for NF-ir neurites, CR-ir neurite pathology did not show a relationship to plaque size, suggesting that physical displacement by Aβ deposits (e.g. DeWitt and Silva 1996; Woodhouse et al. 2005) did not affect CR-ir neurite DN formation. Combined with the higher number of normal-appearing CR-ir neurites and generally fewer CR-ir DNs, this could imply that CR-ir neurites may have a higher capacity for short-term structural plasticity (i.e. avoiding focal injury), or an increased regenerative capability following axonopathy. Both the former (Lee et al. 2006, 2008; Blizzard et al. 2011) and latter (Fenrich et al. 2007) possibilities have previously been shown *in vivo*, suggesting that a combination of the two could potentially mediate the interneuron resistance to AD cytoskeletal pathology seen in this study and others. In addition, the calcium-binding proteins expressed by CR-ir and other interneurons could be neuroprotective, as Ca^{2+} dysregulation is an early feature of Aβ toxicity (Busche et al. 2008; Kuchibhotla et al. 2008; Schwaller 2009). Indeed, subpopulations of pyramidal neurons that express calbindin-D28K in vulnerable regions do not develop NFTs, consistent with a protective role (Greene et al. 2001; Riascos et al. 2011).

In AD, synaptic loss and dysfunction is the earliest identifiable pathological change, likely beginning decades earlier than the onset of cognitive symptoms (Davies et al. 1987; DeKosky and Scheff 1990; Terry et al. 1991; Scheff and Price 2006, 2007; Huang and Mucke 2012; Sheng et al. 2012). To complement the neurite morphology

analysis, this thesis quantified synaptic density near Aβ plaques in human AD cases and transgenic APP/PS1 mice. Specifically, excitatory (VGlut1-ir) and inhibitory (VGAT-ir) presynaptic bouton numbers were analysed in Aβ plaque-heavy cortical areas, and compared to analogous plaque-free areas in the same sections, and control tissue. This study found a general preservation of inhibitory bouton numbers in both human cases and transgenic mice, similar to the previous neurite analysis. In contrast, excitatory boutons were reduced, both in plaque-rich neuropil as expected, but also in plaque-free areas. These changes were apparent even in preclinical AD cases and very young pre-plaque depositing mice, reflecting an early onset of synaptic dysfunction. These findings support previous reports in the literature of a selective glutamatergic decline and a relative sparing of GABAergic terminals (Davies et al. 1998; Bell et al. 2003, 2006, 2007; Hu et al. 2003; Gsell et al. 2004; Calabrese et al. 2007; Dong et al. 2007; Rissman and Mobley 2011).

Additionally, this study analysed bouton morphology in Aβ-rich neuropil. Alongside the excitatory synapse loss, there was a general increase in VGlut1 bouton size in preclinical AD cases and APP/PS1 mice compared to control tissue. A similar pattern was observed for perisomatic synaptophysin puncta in the plaque periphery, whereas inhibitory perisomatic bouton size was significantly smaller in transgenic animals compared to controls. These changes in bouton size likely reflect synaptic remodelling to compensate for the altered excitatory:inhibitory ratio following selective loss of glutamatergic boutons (Palop and Mucke 2010; Isaacson and Scanziani 2011). Larger bouton sizes could reflect a bigger presynaptic vesicle pool with a higher release probability (Wison et al. 2005; Branco et al. 2010). In combination with other altered plasticity responses such as increased inhibitory neuron sprouting (Palop et al. 2003, 2005; Chin et al. 2005), such compensatory

mechanisms may be initially neuroprotective. However, given the long timecourse of AD, prolonged suppression of inhibition in order to match lower levels of glutamatergic signalling in certain highly vulnerable regions such as the hippocampus, could eventually cause aberrant network activity and increased epileptogenesis (Palop et al. 2007; Larner 2010; Tan et al. 2010; Gleichman et al. 2012).

There are a few possible reasons why inhibitory synapses could be spared compared to excitatory synapses. Although APP is expressed ubiquitously, previous studies have shown that Aβ accumulates post-synaptically at glutamatergic (Almeida et al 2005; Snyder et al. 2006; Shankar et al. 2007; Li 2009, 2011) but not inhibitory (Lacor 2004, 2007; Calabrese et al. 2007; Dinamarca et al. 2011) synapses. Moreover, inhibitory presynaptic boutons express different synaptic machinery than excitatory ones. For example, VGlut1-ir boutons are enriched for synapsin-1 and -2, synaptophysin 1, SNAP-25 and syntaxin-1a (Bragina et al. 2007, 2010); while VGAT-ir boutons have higher expression of synaptophysin-2, synaptotagmin-2, synaptobrevin-1, syntaxin-1b and Rab3c (Fox and Sanes 2007; Bragina et al. 2007, 2010, 2011). This not only reflects functional differences in release mechanisms between these classes of synapse, but may confer selective vulnerability on synapses with particular sets of presynaptic proteins to Aβ toxicity and synaptic depression (Varela et al. 1999). More research into the interaction of specific subtypes of presynaptic machinery proteins with Aβ is needed.

Apart from bouton number and size, the level of inhibition in the brain is determined by overall neuron activity. Specifically, neuronal stimulation is an important determinant of GABA production by glutamate decarboxylase (Chattophadyaya et al. 2007; Lau and Murthy 2012). Since GAD activity is driven by overall activity levels

(Hartman et al. 2006), which may be perturbed in the AD brain (Palop and Mucke 2010), it may provide an insight to the functional disruption occurring in prodromal stages of the disease. To assess this possibility, I analysed GAD activity in APP/PS1 mice at various ages. Interestingly, there was an increase in GAD enzyme activity specific to the aged Aβ plaque-rich cortex, not observed in plaque-free areas such as the cerebellum despite similar levels of available GAD. Upon further analysis, it was determined that this increase was due to astrocyte GABA production in APP/PS1 cortical tissue. This may represent a pathologically stimulated increase in the normal contribution of astrocytic GABA to tonic inhibition of neurons (Wu et al. 2007; Lee et al. 2011a,b; Heja et al. 2012). This suggests that boosting overall GABAergic function, particularly at later stages of disease progression when network activity is severely disrupted (Palop et al. 2007; Busche et al. 2008; Larner et al. 2010; Palop and Mucke 2010) may be an effective therapeutic approach. In this regard, selective $GABA_AR$ agonists have been shown to protect cultured neurons from Aβ excitotoxicity (Gu et al. 2003; Lin and Jun-Tian 2003; Louzada et al. 2004; Lee et al. 2005a), as well as promoting non-amyloidogenic APP processing (Marcade et al. 2008).

As discussed in Chapter 1.4, neuronal populations that are particularly susceptible to AD pathology often give rise to long cortico-cortical connections (Hof et al. 1999) that are either later-myelinating in development (Yakovlev and Lecours 1967), or remain incompletely or unmyelinated (Hildebrand et al. 1993; Braak et al. 2000; Bartzokis 2004). Given that these axons usually have to traverse long distances to reach their post-synaptic targets, they could be particularly susceptible to Aβ plaque-mediated cytoskeletal disruptions that affect axonal transport of organelles vital for survival (Vickers 2000; Morrison and Hof 2007; Vickers et al. 2009; Gold et al.

2012; Huang et al. 2012). Similarly, long cortico-cortical association fibres are selectively vulnerable to cytoskeletal pathology and degeneration in APP/PS1 transgenic mice (Delatour et al. 2004; Song et al. 2004; Sun et al. 2005; Adalbert et al. 2009; Chen et al. 2011b; Zerbi et al. 2012). Myelination is known to speed up axonal transport and stabilize the cytoskeleton (Kirkpatrick et al. 2002; Edgar et al. 2004), and to provide trophic support to ensheathed axons (Dougherty et al. 2000; Du and Dreyfus 2002; Wilkins et al. 2003; Fünfschilling et al. 2012; Lee et al. 2012). Therefore, absent or poor myelination of these long axons could also predispose them to metabolic stress (Andrews et al. 2006). To assess myelin damage in AD the AD cortex, a morphological analysis of oligodendrocyte lineage markers and myelinated fibres near amyloid plaques was performed.

Although gross imaging studies have reported white matter loss in AD (Bartzokis et al. 2003; Stricker et al. 2009), little was previously known about grey matter myelination. This thesis has demonstrated a focal loss of myelin associated with fibrillar Aβ plaques in grey matter, highlighting damage to myelinated axons as an important contributor to the loss of cortical integration and brain dysfunction associated with AD. Consistent with the neurite pathology, the greatest degree of demyelination occurred at dense plaque cores in both human AD cases and mouse models, whereas diffuse human Aβ deposits did not result in demyelination. Similarly, oligodendrocyte numbers were significantly reduced at the plaque core in both preclinical and sporadic AD cases compared to controls, whereas there was no change observed in plaque-free human neuropil. In APP/PS1 mice, similar reductions of mature oligodendrocyte numbers were observed, in addition to decreases in the levels of integral myelin proteins. This is in agreement with previous reports of Aβ-mediated oligodendrocyte toxicity *in vitro* (Xu et al. 2001; Lee et al.

2004; Roth et al. 2005; Chen et al. 2006) and alterations in the biochemical composition of myelin *in vivo* (Svennerholm and Gottfries 1994; Roher et al. 2002; Desai et al. 2009). This study also demonstrated an increase in OPC and immature oligodendrocyte numbers that coincided with plaque development in APP/PS1 animals; similar reports of increases in OPCs and decreases in mature oligodendrocytes have been reported in APP/PS1 and 3xTg mice (Desai et al. 2010; Behrendt et al. 2012) and in human cases (Ihara et al. 2010).

In the healthy brain, damaged cortical myelin is actively removed and replaced, a dynamic process known as remyelination (Franklin and ffrench-Constant 2008; Bruce et al. 2010), and we might expect that remyelination and compensation by less affected brain regions could limit the cognitive impact of the focal demyelination observed. In this regard, the present data showing significant increases in cortical APP/PS1 OPC numbers and increased OPC production in the SVZ, combined with recent reports of similar OPC accumulation in human AD cases as well (Behrendt et al. 2012), may indicate a degree of remyelination occuring in AD. On the other hand, Aβ plaques may impair nearby remyelination attempts and predispose neurons to apoptosis (Irvine and Blakemore 2008). Furthermore, oligodendrocyte precursors from later-myelinating cortical regions have reduced myelin turnover and thus a diminished capacity for myelin repair than earlier-myelinating oligodendrocytes (Power et al. 2002; Shen et al. 2008). This could account for the retrogenic pattern of myelin damage observed in AD with later-myelinating regions having a poorer repair response to AD pathology (Reisberg et al. 1999; Braak et al. 2000; Bartzokis 2004). In addition to disruptions in cortical circuitry, focal loss or unravelling of the myelin sheath may also impair local neurite regeneration: myelin-associated proteins, particularly the 66-residue lumenal/extracellular domain of Nogo (Nogo-66), are

potent inhibitors of axonal extension and myelination following CNS injury (Akbik et al. 2012; Borrie et al. 2012). This could drive aberrant sprouting observed around plaque-associated dystrophic neurite pathology (e.g. Vickers et al. 1996; Phinney et al. 1999; Dickson and Vickers 2001).Therefore, therapeutic strategies that seek to enhance OPC differentiation at early stages of AD may prove beneficial as remyelination is neuroprotective (Bruce et al. 2010).This thesis has substantially contributed to our growing understanding of cell-type-specific effects of AD pathology on neurons and their synaptic connections, as well as identifying focal demyelination as a significant contributor to cortical damage in human AD cases. The compensatory mechanisms implied by the findings detailed here may provide novel therapeutic targets in AD, and merit further investigation.

7.1 Conclusions

- **Neurofilament-expressing neurons are selectively vulnerable to dystrophic neurite formation**
- **Calretinin-expressing inhibitory neurons are relatively resistant to dystrophic neurite formation, potentially due to a higher capacity for structural remodelling**
- **Inhibitory VGAT-ir presynaptic bouton numbers are preserved in Aβ plaque-rich neuropil in both human and transgenic mouse cases, but this is accompanied by a decrease in bouton size, particularly near Aβ plaques**
- **Excitatory VGlut1-ir presynaptic bouton numbers are decreased early in AD disease progression in both human AD cases and transgenic mice,**

with a concomitant increase in bouton size, particularly in proximity to Aβ plaques

- **There is an Aβ-associated increase in astrocyte production of GABA in the APP/PS1 neocortex**
- **Cortical Aβ plaques are associated with focal demyelination in human AD cases and Tg2576 and APP/PS1 mice**
- **Cortical Aβ plaques are associated with local depletion of oligodendrocytes in human AD tissue**
- **In APP/PS1 mice, plaque-associated focal demyelination and oligodendrocyte loss was accompanied by increased numbers of OPCs and immature oligodendrocytes, representing possible compensatory mechanisms which could be stimulated therapeutically**

7.2 Experimental limitations

Although the experiments outlined in this thesis make a strong and compelling case for the conclusions that I have made and discussed above, there are nevertheless gaps in some of the experimental designs. These occurred either due to lack of the necessary materials/reagents or simply due to time constraints. In particular, this thesis would have greatly benefited from additional fresh human tissue samples in order to analyse protein/mRNA levels of synaptic (Chapter 4) proteins and how they vary with disease severity (i.e. Preclinical/MCI/end-stage AD cases). Additional post-mortem tissue samples from regions other than the temporal cortex would also have benefitted the analysis of interneuron and pyramidal neurite vulnerability in different regions, with different involvement in AD pathogenesis (Chapter 3). A key omission was the lack of EM studies to complement the synaptic/neurite experiments as this would have further corroborated the present findings of this thesis. This is

definitely something our laboratory is planning on addressing in future experiments. With regards to the very novel and intriguing finding of altered gliotransmission in transgenic APP/PS1 mice, it would have been informative to conduct some additional *in vitro* studies to further elucidate the relationship between Aβ and astrocyte signalling/transcriptional changes.

7.3 Future directions

1. Analyse additional interneuron populations (e.g. PV+, SOM+, as well as neuropeptide expression) in both transgenic cases at various ages, as well as in aged control, presymptomatic (preclinical AD), MCI and end-stage AD cases. Of particular interest would be to compare different regions such as frontal, temporal, primary sensory cortices, as well as subcortical regions (e.g. CBF system). This will provide further understanding of the cellular vulnerability underpinnings of AD and likely shed more like on the circuits involved and potential compensatory responses (cf. Ikonomovic et al. 2003) from less affected regions/circuits.
2. Conduct further analysis on fresh tissue samples of APP/PS1 mice and human cases to assess if the synaptic changes reported here are borne out by decreases in mRNA levels and/or other post-transcriptional modifications.
3. Conduct *in vitro* astrocyte culture experiments to tease apart the mechanism of Aβ induced alterations in gliotransmission. In particular, the mechanism of release and the responses to different concentrations/formulations of Aβ will be examined. Also of interest would be to analyse global changes in gene/mRNA expression in astrocytes (particularly genes regulating the exocytotic/signalling pathways) following Aβ administration.

4. In a similar vein, *in vitro* oligodendrocyte mono- and co-cultures would provide more clues as to the role of Aβ signalling (both physiological and pathological) on myelination. Of particular interest would be to assess if there are changes in ACh receptor expression/signalling in oligodendrocytes. Although it is known that Aβ is less toxic to OPCs *in vitro*/*in vivo*, it will be informative to see if it has effects on their remyelination/myelination potential as well.

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Appendix A

0.01M PBS, pH 7.4

100mL	9% NaCl (90g of NaCl (Sigma) per 1L Milli-Q® water)
40mL	Na_2HPO_4 (BDH Laboratory supplies, Poole, UK) (28.4g per 1L Milli-Q® water)
10mL	$NaH_2PO_4.2H_2O$ (Sigma) (31.2g per 1L Milli-Q® water)
850mL	Milli-Q® water

18.0% Sucrose Solution

180g	Sucrose (Sigma) dissolved in 1L 0.01M PBS

30.0% Sucrose Solution

300g	Sucrose (Sigma) dissolved in 1L 0.01M PBS

Tissue Storage Solution

0.01M PBS

0.1% Sodium azide (Sigma)

0.25% Potassium permanganate

0.125g	$KMnO_4$ (BDH) in 50mL 0.01M PBS

1.0% Potassium-metabisulphite and oxalic acid

0.5g	$K_2S_2O_5$ (BDH)
0.5g	Oxalic acid (Analytic Univar Reagents, Victoria, Australia)

Dissolve in 50mL 0.01M PBS

0.0125% thioflavine s

0.00625g	thioflavine s (Sigma) in 50mL 0.01M PBS

0.3% Triton/PBS

600μL Triton X (Sigma)

200mL 0.01M PBS

0.01M Citrate Buffer

2.94g Trisodium citrate (Sigma) in 800mL Milli-Q® water

Dissolve and adjust to pH 6 with 0.1M citric acid, then make up to 1L with Milli-Q® water.

0.1M Citric acid

10.5g Citric acid (Sigma) in 400mL Milli-Q® water

Dissolve and adjust to pH 6.0 with 2M NaOH, then make up to 1L with Milli-Q® water.

4% Paraformaldehyde (PFA)

40g PFA (Sigma)

40g Sucrose (Sigma)

100mL 9% NaCl

400mL Na_2HPO_4

500mL $NaH_2PO_4.2H_2O$

Heat while stirring until dissolved in a fume hood.

1.0% Hydrogen peroxide in methanol

1mL 30.0% H_2O_2 (Sigma) diluted in 30mL methanol

Silver Solution

100mL Milli-Q® water

0.1g NH_4NO_3 (Sigma)

0.1g $AgNO_3$ (Sigma)

0.3mL 4% NaOH (Sigma)

Developer Stock 'A'

5.0g	Na_2CO_3 (Sigma)
100mL	Milli-Q® water

Developer Stock 'B'

0.2g	NH4NO3 (Sigma)
0.2g	$AgNO_3$ (Sigma)
1.0g	tungstosilicic acid (Sigma)
100mL	Milli-Q® water

Immunoblotting Solutions:

NuPAGE® MOPS SDS Running Buffer (20x) pH 7.7

104.6g	MOPS
60.6g	TrisBase
10.0g	SDS
3.0g	EDTA

Adjust final volume to 500mL with Milli-Q® water. (recipe from Invitrogen, Eugene, OR)

NuPAGE® Transfer Buffer (20x) pH 7.2

40.8g	Bicine
52.4g	Bis-Tris (free base)
3.0g	EDTA

Adjust final volume to 500mL with Milli-Q® water. (recipe from Invitrogen, Eugene, OR)

NuPAGE® LDS Sample Buffer (4x) pH 8.5

0.666g	Tris HCl

0.682g	Tris Base
0.8g	LDS
0.006g	EDTA
4.0g	Glycerol
0.75mL	SERVA® Blue G250 (1% solution)
0.25mL	Phenol Red (1% solution)

Adjust final volume to 10mL with Milli-Q® water. (recipe from Invitrogen, Eugene, OR)

Synaptosome and Percoll Gradient Solutions:

0.32M Sucrose

10.95g	Sucrose (Sigma)
100mL	Milli-Q® water

Krebs Ringer Phosphate Buffer pH 7.4

36.82mg	NaCl (Sigma)
177.1mg	KCl (Sigma)
70.47mg	$CaCl_2$ (Sigma)
144.3mg	$MgCl_2.6H_2O$ (Sigma)
1.1g	$NaH_2PO_4.H_2O$ (Sigma)
1.0g	Dextrose anhydrous (Sigma)

Dissolve salts in 400mL of Milli-Q® water and adjust pH to 7.4. Make up to 500mL.

0.2M EDTA

7.45g	EDTA (Sigma)

Dissolve in 75mL of Milli-Q® water and adjust pH to 7.4 with Tris base. Make up to 100mL.

Gradient Buffer (4x)

109.54g Sucrose (Sigma)

606.0mg Tris base (Sigma)

Dissove in ~180mL of Milli-Q® water, add 5.0mL 0.2M EDTA stock and dilute to ~240mL. Adjust pH to 7.4 if necessary. Dilute to a final 250mL.

Sucrose/EDTA Buffer

Dilute 50mL of 4xGradient Buffer to 200mL with Milli-Q® water.

50mM DTT

154mg Dithiothreitol (Sigma)

10mL Milli-Q® water

Myelin extraction solutions:

1M Tris-Cl

121.1g Tris base in 800ml of dH_20

pH to 7.45 with 1M HCL (~65ml of HCL)

Add dH_2O to make to 1L

100mM Na_2EDTA

33.6g/L of anhydrous Na_2EDTA (Mw=336.21)

to make 100ml, divide mass by 10 respectively (i.e. 3.36g in 100ml dH_2O)

Tris-Cl Buffer Solution

To 100ml of H_20 add:

4ml 1M Tris-Cl, pH7.45 at 25°C (i.e. 20mM final)

4ml 100mM Na_2EDTA (2mM final)

0.031g DTT (optional)

Adjust to pH7.45 if required (with 1M Tris-Cl or 1M HCl)

Add dH_20 to 200ml

Add protease inhibitors

Store up to 1-2days at 4°C or longer at -20°C

1M Sucrose solution:

To 100ml of dH_20 add:

68.46g sucrose (1M final)

4ml 1M Tris-Cl, pH7.45 at 25°C (i.e. 20mM final)

4ml 100mM Na_2EDTA (2mM final)

0.031g DTT (optional)

Adjust to pH7.45 if required (with 1M Tris-Cl or 1M HCl)

Add dH_20 to 200ml

Add protease inhibitors

Store up to 1-2days at 4°C or longer at -20°C

0.30M Sucrose solution

Dilute 1M Sucrose solution with Tris-Cl buffer solution (v/v) 0.30/0.70

0.83M Sucrose solution

Dilute 1M Sucrose solution with Tris-Cl buffer solution (v/v) 0.83/0.17